ANALYSIS OF SELENIUM TOXICITY ON REDUCED THIOL CONTENT

By

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ABSTRACT

Enterobacter sp. YSU a multimetal resistant strain, grew when exposed to 40 mM selenite by reducing it to elemental selenium and thus detoxifying it. Growth curves established in M-9 minimal medium showed that *Enterobacter* sp. YSU grew in the presence of 40 mM selenite only when supplemented with L-cysteine and died when exposed to selenite in the absence of L-cysteine. A previous study involving M-9 minimal medium suggested that when selenite concentration was higher than the sulfate concentration selenite could enter the *Enterobacter* sp. YSU cells through two pathways: a specific pathway and a non specific pathway (sulfate permease channel). The presence of L-cysteine prevents the entrance of selenite through non specific pathway. The actual toxicity mechanism of the selenium oxyanion, selenite (SeO_3^{2-}), is unknown. To determine if the interaction of selenite with the reduced thiols is involved in the toxicity mechanism and the reason for oxidative stress, the RSH content of *Enterobacter* sp. YSU was assayed. This strain was grown under sensitive (no L-cysteine) and resistant (presence of L-cysteine) conditions. Selenite was added during the early log-phase. Cells were harvested before and after the exposure to selenite, lysed and reacted with RSH specific derivatizing agent 5, 5' – dithiobis (2-nitrobenzoic acid) for thiol analysis. HPLC was performed for specific quantification of reduced glutathione. The derivative formed represented the level of oxidative stress. Cells were also harvested for Bradford assay to determine the cell protein concentration. It allowed for the RSH content to be normalized to total cell protein. Upon exposure of selenite, the RSH content of the cells grown under sensitive conditions decreased markedly. In the presence of L-cysteine, only a small fraction of RSH content became oxidized. Total protein concentration of the sensitive

cells declined as well, as compared to the resistant cells. HPLC analysis showed that there was a decrease in reduced glutathione in sensitive cells. However, when the reduced thiol and glutathione levels were normalized to total cell protein, the results were not significant. This observation was probably caused by the rapid lysis of the sensitive cells in to the culture media. Using the data obtained by measurement of protein both in the cells and in the culture media could be significant.

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LIST OF SYMBOLS AND ABBREVIATIONS

EFPC……East Fork Poplar Creek

(GSH-Px)……..Glutathione peroxidase

Se-Cys……Selenocysteine

TxnRd……...Thioredoxin reductase

TPN……...Total Parenteral Nutrition

DNA……....Deoxyribo Nucleic Acid

RNA……..….. Ribo Nucleic Acid

Cys……...Cysteine

($GS\text{-}Se^-$)……..Selenopersulfide

(GS-Se-SG)……...Selenoglutathione

(RS-Se-SR)……..Selenotrisulfides

GSSG……...……Oxidized glutathione

GSH…….....Reduced glutathione

NADPH……..……Nicotinamide Adenine Dinucleotide Phosphate

$NADP^{+\cdots}$……..……..Nicotine Adenine Dinucleotide Phosphate

RSH……..….Reduced Thiol

H_2SeO_3…….. Selenious acid

SOD……...…Superoxide dismutase

µm……...Micrometer

A…….Angstrom

C18……... Octa Docyl Silane

nm……..…….…….. nanometer

µL……...microliter

NaOAc……..Sodium acetate buffer

Mg……..…Milligram

mL……...… Milliliter

µL……...Microliter

µg…….Microgram

mM……....Millimolar

M……. Molar

Min……Minutes

DTNB……...5, 5' – dithiobis (2-nitrobenzoate)

EDTA……...Ethylenediaminetetraaceticacid

Tris……..Tris (hydroxymethyl) aminomethane

SDS...……..Sodium Dodecyl Sulfate

HPLC……... High Performance Liquid Chromatography

GSH……Glutathione

NCNS……No Cysteine, No Selenite

NCS…….No Cysteine, Selenite

CNS…….Cysteine, No Selenite

CS……..Cysteine, Selenite

^{0}C…….……..Degree Celsius

PBS……...……...……Phosphate buffered saline

sp. ……..Species

RSH……...Thiol

Chapter I: Introduction

1.1. The Oakridge Y – 12 Plant

The Oak Ridge Y-12 Plant is situated adjacent to the city of Oak Ridge, in Anderson County, Tennessee. It was constructed as part of the Manhattan Project in 1943 with a mission to separate fissionable isotopes of uranium (U-235) by the electromagnetic process.[1] Its job was to process uranium for the first atomic bomb (which was dropped on Hiroshima Japan in 1945).[2, 3]

Over the ensuing years, Y-12 plant has become a highly sophisticated nuclear weapons component manufacturing and development engineering organization. The mission of Y-12 changed during the Cold War, it focused on processing lithium to make hydrogen bombs which required large amounts of mercury (key element used to capture enriched lithium by separating the lithium isotopes)[17] which was not contained and approximately 920,000 kg were spilled into the surrounding environment.[4] About 3000 µg/g of Hg was found in East Fork Poplar Creek (EFPC) floodplain soils in various physiochemical forms such as Hg^0, dissolved ionic Hg, fine Hg particles attached to suspended matter, mercuric oxide, organic Hg covalently bound to and mercuric sulfide.[9, 10,] As per severe contamination of Hg, this site was placed on the National Priority List to undergo remediation.[11 ,12] Other wastes were also disposed in different places of the area.

Four unlined impoundments (S-3 ponds) were constructed at the western margin of the Oak Ridge Y-12 plant in 1951, near the origins of Bear Creek and East Fork Poplar Creek (EFPC). Each had a capacity of 9.5 million liters. These ponds received various liquid (acidic) wastes generated from uranium operations and other heavy metals. They

lacked coverings and linings to allow the waste to evaporate or decontaminate as it passed through soil. Instead the wastes passed in to the nearby creek and thus contaminated them. The waste discharge to these ponds was ceased in 1983 and the remaining wastes were treated. Liquid was drained, followed by tilling and capping of the S-3 ponds.[8, 5]

Current Y-12 missions include receipt, storage, and protection of uranium and lithium materials and parts.[2, 3] As large amounts of heavy metals were used during the production of nuclear materials and due to their improper disposal it is assumed that some of these heavy metals have been lost in the environment contaminating the nearby areas.[7, 8] Successful treatment would provide the opportunity for considering replacement at the site.

1.2. Bacterial Strain

1.2.1. *Stenotrophomonas maltophilia*

Stenotrophomonas maltophilia Oak Ridge O2 (*S.maltophilia* O2) (ATCC # 53510) is a bacterial strain resistant to many metals. It was isolated from East Fork Poplar Creek, TN. During the process of 16sRNA sequencing (used to identify bacterial strains) it was discovered that the working strain of *S.maltophilia* was actually an *Enterobacter* species. It was confirmed to be an *Enterobacter* species by phylogenetic tree analysis and biochemical tests. As it was discovered at YSU the strain was named as *Enterobacter* sp. YSU.

1.2.2. *Enterobacter* sp. YSU

Enterobacter sp. YSU belongs to the family Enterobacteriaceae. It is a genus of gram-negative, facultative anaerobic rod-shaped bacteria.[111] *Enterobacter* sp. YSU showed resistance to mercury (Hg (II)), cadmium (Cd (II)), zinc (Zn(II)), gold (Au(III)), arsenic (As(III)) and selenite (Se(IV)).[100]It showed growth in the presence of lead, cadmium, copper, platinum, mercury, chromium, gold, silver and selenium salts.[100-101] It could detoxify the metal salts by converting them into insoluble precipitates. *Enterobacter* sp. YSU could reduce the toxic forms of selenium (selenite) into elemental selenium and thus detoxify it.

Chapter II: Selenium and Glutathione

2.1. Selenium

Selenium is a normally occurring trace element. It is found in sulfide ores, bearing an atomic number 34, and belongs to group VI A in the periodic table. It is characterized as a metalloid having the properties of both a metal and nonmetal.[13]It is essential for humans and animals in small concentrations but becomes toxic in higher concentrations.[14, 18] Selenium is present abundantly in igneous rocks, some sedimentary rocks and fossil fuels. It also found in shale, sandstone, limestone, coal, soil, surface water and vegetation.[19]

As selenium belongs to the same group as that of sulfur (VI A elements), they share many chemical and biochemical properties.[37-38] The four oxidation states of selenium are selenate [Se (VI)] (an analog of sulfate)[35] , selenite [Se (IV)], elemental selenium [Se (0)] and selenide [Se (II)], which can occur as organic or inorganic selenides.[35] Selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) are toxic water soluble species that bioaccumulate. Elemental selenium (Se^0) is essentially nontoxic and insoluble in water. Though selenide (Se^{2-}) is both highly reactive and toxic it is readily oxidized to Se^0.[13] Elemental selenium and selenide are the least mobile reduced forms of selenium. They are the predominant species at low pH, especially in water logged soil conditions, rich in organic matter.[57, 63]Selenide can exist as metal selenide or organoselenium compound, while elemental selenium can reside in differing allotropic forms.[57, 59-60]In environmental samples any or all of the four oxidation states of Se may be present, each with its unique mechanism of retention.[57, 61]In aerated soils and aquatic environments, selenium occurs predominantly in the form of inorganic selenite and selenate oxyanions (SeO_3^{2-} and SeO_4^{2-}), which are freely available to living organisms [14, 34] whereas elemental selenium

is dominant in anaerobic sediments.[18] The organic selenium compounds (selenomethionine, selenocysteine) are the major selenium species in cereal and in vegetables.[24]

Three forms of elemental selenium have been described, a red amorphous form, a black amorphous form, and a grey hexagonal form (most stable form). The red and black amorphous allotropes most likely occur in soils. At temperatures above 30°C, red amorphous Se^0 gradually changes into the black amorphous form. This form is then slowly transformed into the more stable, grey hexagonal allotrope or it is reoxidized, depending on the redox conditions and the pH of the soil. Oxidation can occur by the action of microorganisms or through inorganic reactions.

It was demonstrated that selenate is not absorbed to soil whereas, selenite is absorbed rapidly and is resistant to leaching but represents a potential long term source of Se to ground water. [57] The concentration and speciation of selenium in soil depends on the dynamics of pH, solubility, oxidation – reduction potential (ORP), complexing ability of soluble and solid ligands as well as reaction kinetics.[57, 60, 62-64]

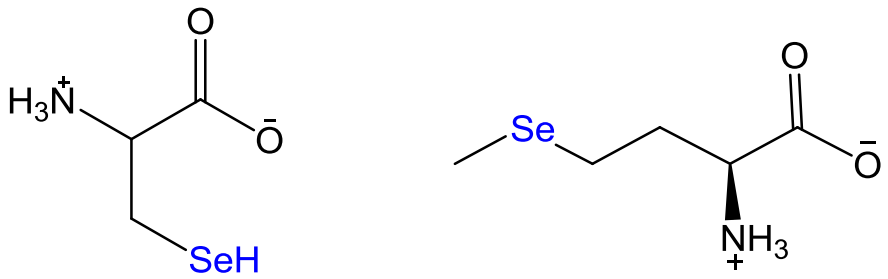
2.1.1. Selenium uses

Selenium has an essential nutritional role in all living organisms. It is required for normal function, growth and reproduction.[19] It is required for synthesis and metabolism of thyroid hormone. The thyroid gland has the highest Se content per mass unit similar to other endocrine organs and the brain.

Selenium is covalently incorporated into several selenoproteins such as the families of glutathione peroxidases, thioredoxin reductase and deiodinases.[23] Thioredoxin reductase (TxnRd) and glutathione peroxidase (GSH-Px) are amongst the most widely

found selenoproteins in mammals. Selenium incorporation in to proteins occurs majorly in the form of selenocysteine.[19, 29]

Selenocysteine (Se-Cys) is the 21st natural amino acid occuring in the genetic code. Selenomethionine, the second Se-containing amino acid incorporated in to the proteins at methionine position. Selenocysteine is functionally active and its biosynthesis is regulated and is encoded by the UGA-codon.[32] Selenomethionine is not directly incorporated into proteins and has no specific function in proteins.[23] The organic selenides can be incorporated in to sulfur's biochemical pathways, forming selenomethionine and selenocysteine analogs of methionine and cysteine.[35]



Seleno-cysteine Seleno-methionine

Figure 1. Structures of Seleno-cysteine and seleno-methionine

The biochemical function of selenium is recognized by its role as a component of glutathione peroxidase. Currently many chemical forms of Se are in medical use.[23] It has been determined to be effective in preventing vitamin E deficiency disorders in animals.[31] The possible use of selenium in cancer prevention and in chemotherapy has been suggested. Selenium in the form of selenoprotein (glutathione peroxidase) is used as an antioxidant in the treatment of carcinogenesis and heart diseases.[19]

Selenite, selenate and selenomethionine are added to Total Parenteral Nutrition (TPN) and intravenously administered for treatment and prevention of selenium deficiency.[50, 51]

Selenium is involved in several major metabolic pathways as well as antioxidant defense systems. It is involved in the prevention of numerous chronic illnesses such as specific cancers and neurodegenerative diseases.[21] Numerous mutagenic and anti mutagenic effects of selenium have been reported.[30] Furthermore, it participates in the regulation of immune function.[23] Dietary supplementation of selenium is claimed for the prevention of angina, arthritis, cancer, cerebrovascular disease, aging, infections and cataracts in humans. Patients with HIV often exhibit low selenoprotein concentrations in their cells. Thus AIDS patients are recommended to take selenium supplements to allow their cells to continue to produce selenoproteins. Deficiency of selenium leads to abnormalities in many functions of the brain, heart, liver, striated muscle, pancreas and genital tract. Deficiency of glutathione peroxidase causes increased production of superoxides and hydrogen peroxidase leading to atherogenesis.[22]

At certain times, sulfur found in sulfide minerals and pyritic coal deposits are substituted with selenide.[20] Selenium has varied industrial and commercial uses. It is used in semiconductor research, in glass manufacturing, in photoconductors, semiconductors, photoelectric cells, rectifiers, electron emitters, pharmaceutical products. It is a catalyst in Kjeldahl digestions (cupric selenite), also used as a chemical reagent (potassium selenate). It is used for treatment of eczemas and fungal infections in veterinarian remedies, as an antidandruff agent in shampoos for human use (selenium disulfide). In

radiologic diagnostic procedures the radioactive nucleotides of selenium are used as biologic tracers.[19, 24]

2.1.2. Exposure to selenium

The primary pathway of exposure of selenium to the general population is through food, followed by water and air.[24] According to US-EPA and in Canada the maximum allowable concentration of selenium in drinking water is 10 µg/ L. [97, 98] Selenium levels in groundwater and surface water range from 0.06 to about 400 µg/L but can reach up to 6000 µg/L. The levels of selenium in air ranges from 0.1 to 10 ng/m^3. Daily intake of 0.9 µg/kg of body weight of selenium in adult's and1.7 µg/kg of body weight of selenium in infants is recommended.[98]

Human activities, such as coal mining, fuel refining, industrial uses of selenium (e.g., in photocopy machines, electronics, glass manufacturing, chemicals, and pigments), affect the biological availability of selenium.[14, 25] These activities cause major concerns within the environmental regulatory agencies. The valence state affects toxicity and bioavailability of selenium.[19]

2.1.3. Toxicity of selenium

Selenium is an essential micronutrient for humans and animals but is toxic at high levels of exposure. [35, 40] It can be highly toxic depending on its concentration and speciation.[71] Se has the lowest toxic-to-essential dietary exposure ratio compared to any other essential element.[57- 58] In sediments and soils, selenium occurs in higher concentrations, which is a serious threat to the environment. It is also the major source of contamination in many anthropogenic activities such as irrigated agriculture, petroleum refining, fossil fuel combustion and mining operations. Thus, many bioremediation and

geochemical process have been implemented to remove Se oxyanions from the seleniferous soils and sediments.

The toxicity depends on the chemical form of selenium.[24] At elevated concentrations, the higher valence states of Se (VI) and Se (IV) are toxic and can cause severe poisoning of fish and waterfowl in contaminated environments.[15] For humans and most other mammals, the toxicity of selenium increases in the order of selenite – selenate.[33] Selenate and selenite bioaccumulate and can be toxic even at relatively low concentrations.[25] The death and deformation of waterfowl in California's San Joaquin Valley was identified due to Se contamination of agricultural drainage water. [25- 28] The oxyanions of Se were implicated as the cause for waterfowl mortalities in the Kesterson wildlife refuge, a man made salt marsh which received the agricultural drainage waters of the San Joaquin valley. [37]

Selenite-sensitivity in bacteria is mainly due to the formation of reactive oxygen species (ROS). Highly toxic substances such as hydrogen peroxide, H_2O_2 and superoxide, O_2^- are produced by the reaction of glutathione peroxidases with selenite causing damage to cell membranes and DNA. These oxygen species cause oxidative stress which is responsible for the toxicity of selenite.[18]

Abnormalities may occur in the development of various plants and animals at higher concentrations of selenium. Structural modifications and various deformities have been noted especially in keratin formed tissues (hooves, horns, hair, feather, beaks and nails), in which significant quantities of selenium may accumulate. In this regard the selenium toxicity is attributed to its capacity to replace sulfur in proteins or other sulfur containing biomolecules.[71] Changes in the hair and nails have been reported in China due

to chronic selenium poisoning which resulted from excessive environmental exposure to selenium. The acute ingestion of selenious acid is extremely fatal, preceded by stupor, hypotension and respiratory depression. Garlic odor of the breath is an indication of excessive selenium exposure as a result of the expiration of dimethyl selenide.[24]

As sulfur and selenium belong to the same group they share some properties. In this regard, sulfate has been identified as an antagonist for selenate toxicity and transport in various microorganisms.[36-37, 39] Dysfunctional enzyme formation is probably strongest implicated cause of selenium toxicity. Significant loss in enzyme function would result from the synthesis of polypeptides in which some or all of the sulfur amino acids had been replaced by their selenium analogs. The toxic effects of organic selenium are thought to be due to the physical differences between sulfur and selenium molecules.[35, 36]

Study was conducted by Kiffney and Knight in 1990 [35] on the bioconcentration and toxicity of selenate, selenite and seleno-L-methionine in the cyanobacterium *Anabaena flosaquae*, a filamentous nitrogen fixing bacteria. Based on their investigation, it was indicated that seleno-L-methionine is more toxic (approximately 30 times) than selenate and selenite, while the inorganic forms are equally toxic.[35]

Low serum levels of Se and diminished GSH-Px activity have been reported in patients receiving long term parenteral nutrition that does not include Se.[33] It is reported that in the presence of sulfhydryl groups like glutathione (GSH) or cysteine (Cys) in TPN selenite inhibits the activities of DNA and RNA polymerases and enhance hemolytic actions.[50, 52-53] It has also been reported to induce various types of cellular damage in the presence of sulfhydryl groups.[50, 52-56] As it generates ROS, it was found to cause vascular endothelial cellular damage. During selenium deficiency, the activity of glutathione

peroxidase with the ROS scavenging action may be decreased, and thus the toxicity of the compounds generating ROS may be intensified. However, it is not known whether the clinical concentrations of selenite, selenate or selenomethionine generates ROS when it coexists with Cys or GSH.[50]

2.2. Glutathione

Thiols occur in various natural sources and are by-products of many industrial processes.[75] Thiol is a compound which contains a sulfur-hydrogen bond (-SH). As it is the sulfur analog of an alcohol group (-OH), it is referred to as either thiol or sulfhydryl group. It is also known as a mercaptan. Low molecular weight thiols have strong odor. They are widely used as scents, flavors, or as metabolites in environmental, food and pharmaceutical studies.[75] The hydrogen atom of many sulfur containing antioxidants (-SH) can act as an electron acceptor for neutralizing free-radicals. Free radicals are the molecules containing unpaired electrons which are highly reactive and might cause oxidative damage. Cysteine and glutathione are examples of thiols that can reduce free radicals by hydrogen donation. Amongst the various biological mechanisms involved in protecting cells against oxidative stress, the thiolic group in glutathione has the main role in the endogenous defense of the body.[77, 81-82]

Glutathione (γ-L-glutamyl-L-cysteinyl-glycine) (GSH) is an intracellular thiol.[85] It is an important tripeptide which can be synthesized from amino acids L-cysteine, L-glutamic acid and glycine. The thiol (SH) group of cysteine in glutathione serves as the proton donor and is responsible for glutathione's biological activity. Glutathione is involved in antioxidant cellular defense against the toxic effects of reactive oxygen species such as free radicals and peroxides. It is important in protection against drug

toxicity and also maintains many sulfhydryl proteins in their reduced state which is a requirement for their normal functioning. [80] Reduced glutathione (GSH) is the main non – protein thiol species, present in all animal cells, plants and in bacteria.[76-77]

Glutathione exists in two forms, reduced (GSH) and oxidized (GSSG). In the reduced state glutathione donates the reducing equivalent ($H^+ + e^-$) to other unstable molecules, such as reactive oxygen species and thus inactivates them. It readily reacts with another glutathione to form glutathione disulfide (GSSG). GSSG can be converted back to its reduced form by glutathione reductase.

Most extensively glutathione occurs in the reduced form since the enzyme which reduces it from its oxidized state, glutathione reductase, is essentially active and inducible upon oxidative stress. Glutathione acts as an electron acceptor and reduces any disulfide bond formed within cytoplasmic proteins to cysteines. Thus it gets converted to its oxidized form glutathione disulfide (GGSG). The ratio of its reduced and oxidized form is a measure of cellular toxicity. An increased GSSG-to-GSH ratio is considered indicative of oxidative stress. The imbalance between oxidants and antioxidants as well as the depletion of antioxidant defense results in oxidative stress.[76] Deficiency in glutathione levels leads to various pathologies like renal insufficiency, cardiovascular disturbances.[76, 84]

2.2.1. Glutathione as an antioxidant

Substances which can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition are called Antioxidants. Natural defenses against free radicals are present in organisms in the form of antioxidant enzymes, such as superoxide

dismutase (which neutralizes superoxide) and catalase (which neutralizes hydrogen peroxide). Organisms also synthesize non-enzymatic antioxidant molecules such as coenzyme Q and glutathione.

Glutathione, glutathione peroxidase and glutathione reductase constitute the glutathione system which is a key defense against hydrogen peroxide and other peroxides. Glutathione peroxidase (GPx) enzyme occurs in four forms: (1) Cystolic glutathione peroxidase (cGPx) (2) Phospholipid hydroperoxide glutathione peroxidase (phGPx) (3) Plasma glutathione peroxidase (pGPx) (4) Gastro-intestinal glutathione peroxidase (giGPx).

Glutathione peroxidase contains selenium. It is an important enzyme for processes that protect lipids in polyunsaturated membranes from oxidative degradation.[24] Selenium in the diet can increase glutathione peroxidase levels thus selenium has antioxidant properties and is considered an essential dietary requirement. Selenium deficiencies results in depletion of glutathione peroxidase.

Glutathione peroxidase or catalase helps the enzyme superoxide dismutase to neutralize hydrogen peroxide. The enzyme glutathione peroxidase eliminates hydrogen peroxide by utilizing reduced glutathione.

$$2\ GSH + H_2O_2 \longrightarrow GSSG + 2\ H_2O$$

Glutathione reductase then adds hydrogen's to the oxidized glutathione to regenerate reduced glutathione. A high GSH/GSSG ratio indicates a high level of reduced glutathione available for antioxidant activity.

Glutathione participates in drug detoxification and amino acid transport in γ-glutamyl cycle by acting as a free radical scavenger.[76, 79] It plays a fundamental role in various metabolic and biochemical reactions such as DNA synthesis and repair, protein synthesis, amino acid transport and enzyme activation. It is essential for the proper functioning of immune system, the nervous system, the gastro intestinal system and lungs. It regulates apoptosis thus maintaining the control of the immune system.

Excess of free radicals, reactive oxygen and nitrogen species causes oxidative damage of macromolecules.[83, 76] Glutathione serves to protect tissues from damage by acting as a reducing agent in the metabolism of hydrogen peroxide, organic peroxides and free radicals. It deactivates toxic substance and carcinogenic metabolites by forming conjugates with them.

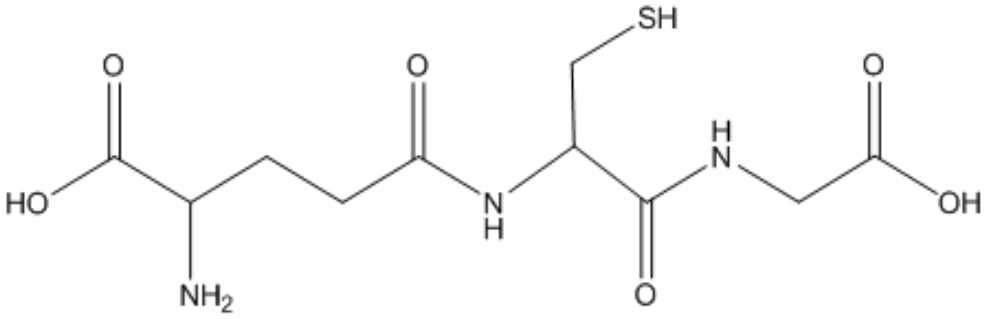


Figure 2. Structure of glutathione

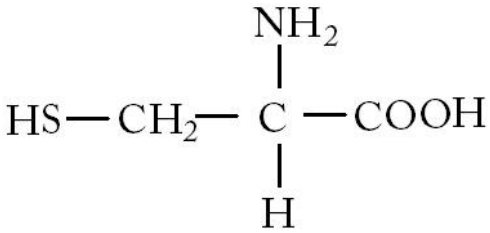


Figure 3. Structure of cysteine

2.3. Use of microorganisms in bioremediation

Chemical detoxification of metal and metalloid-polluted sites is very expensive and often results in secondary effects in the environment.[14] Thus, there is a need to find more sustainable biological solutions. Use of phototrophic microorganisms belonging to the group containing the purple bacteria have been shown to be particularly resistant to a variety of metal and transition metal oxyanions, including selenium. This resistance is attributed to the capacity of the organisms to reduce Se oxyanions to their elemental ground state, which is poorly soluble and thus less toxic than the initial oxyanions.[14] Soluble selenate [Se (VI), SeO_4^{2-}] and selenite [Se (IV), SeO_3^{2-}] reduction to Se^0 converts selenium in to an insoluble mineral form. In soils and sediments, this type of transformation is primarily mediated by microorganisms. [37, 40-43]

Microbial populations that reduce soluble selenate (SeO_4^{2-}) to insoluble elemental Se were found.[25] Microorganisms play a crucial role in the global cycling of selenium through oxidation, reduction, methylation and demethylation. As Se oxyanions undergo microbial transformations, this phenomenon is potentially useful in bioremediation strategies, including 1) bioreduction of soluble selenate (SeO_4^{2-}) and selenite (SeO_3^{2-}) to insoluble forms such as elemental selenium (Se^0) or to assimilated organic forms such as selenoamino acids 2) reduction and methylation, which yield volatile forms, primarily dimethyl selenide. [25, 28, 57, 68-67] Microbes which can reduce these selenium oxyanions are not restricted to any particular group or subgroup of prokaryotes and examples are found throughout the bacterial domain. However in some species, including *Thauera selenatis, Sulfurospirillium barnesii, Enterobacter cloacae* and *Aeromonas hydrophila,* the reduction of selenate is linked to the respiratory electron transfer chain.[34] Members of the

family Enterobacteriaceae were dominant among the selenate reducing isolates from the site containing high Se levels.[57] Of these, *Enterobacter cloacae*, is involved in the reduction of selenate and selenite to elemental selenium using NO_3^- and SeO_4^{2-} as terminal electron acceptors under anaerobic conditions.

Anaerobic Se-respiring bacteria can use Se (VI) and Se (IV) as terminal electron acceptors and precipitate elemental selenium granules. [37, 40, 44-45] Aerobic and phototrophic Se-resistant bacteria can also catalyze the reduction of selenium oxyanions to form insoluble Se (0) particles.[14, 18, 40, 46-47] Reduction of selenium oxyanions by microbes generates red elemental selenium with either crystalline or amorphous structures.[25, 40]

Respiratory reduction of selenate to elemental Se is one of the mechanisms whereby bacteria in sediments remove Se from overlying waters.[37, 57] whereas, for many organisms reduction of soluble selenate and selenite to insoluble elemental selenium is a toxicity resistance mechanism, some will actually use the selenate as an electron acceptor for energy production.[57, 68]

Since selenium in contaminated sites generally exists as the oxyanions, selenite or selenate, the ability of bacteria to reduce these oxyanions to insoluble elemental selenium may be a useful tool for eliminating dissolved selenium. The dissolved selenium that is reduced to elemental selenium can then be removed by filtration. The fate and transport of selenium by microorganisms is influenced by oxidation and reduction of selenium.[28]

2.4. Transport of Selenium

Bacteria contains specific systems to uptake, transport, store, scavenge and to remove elements. Certain elements including selenium utilize these systems which are meant for similar elements and chemical species, to get in to the cells. Studies with M-9 minimal medium has shown that when the concentration of the selenite is higher than the concentration of sulfate, selenite entered into the cells of *E.coli* using two pathways, either the specific pathway (meant for selenite) or the non specific pathway (sulfate permease channel).[99] *Enterobacter* sp. YSU could reduce selenite and selenate into elemental selenium.

In the specific pathway which is unknown, selenium gets into the cells intentionally and is incorporated into the proteins as part of the amino acid selenocysteine. It also gets incorporated in to glutathione peroxidase, specific tRNA molecules and formate dehydrogenases (FDH) as selenocysteine. Selenium uptake takes place as an oxyanion (selenate SeO_4^{2-}). In the non specific pathway, selenate is thought to enter the cell through sulfate permease channel using *cys*A, *cys*U and *cys*W genes as observed in *E.coli.* and gets incorporated as selenocysteine unintentionally instead of L-cysteine. Changes in these genes results in selenate resistance. Selenite might as well use this pathway, but it is not confirmed that the sulfate permease pathway is the only way as when it was inhibited the selenite uptake did not stop. Thus, there could be an existence of alternative selenite transporter.[99, 106]

Selenium uptake is a complicated pathway since selenium is both necessary for cell growth and also toxic at higher concentrations. Under toxic conditions it can either

get detoxified or get incorporated into proteins, tRNA molecules or FDH. A resistant bacterium detoxifies selenate and selenite by reducing them to elemental selenium or by methylation. Detoxification by reduction is shown by a strain of *Stenotrophomonas maltophilia*, collected from selenate and selenite contaminated site. This bacterium deposits the elemental selenium near the cell surface and the surrounding growth medium. [2]

Thorough understanding of the complete metabolism and biochemical fate of selenium is necessary to know the toxicity mechanism of the element and its effects on various enzymes and metabolic pathways involving selenium.

2.5. Metabolism of selenium

The reduction of the selenate can take place in the periplasm. *E.coli* possesses periplasmic nitrate reductase (napA) which shows this activity. After selenate enters the cell it can be reduced to selenite by the nitrate reductases A and Z (narGHIJ and narZUWV respectively). Reduction of selenite to elemental selenium is not catalyzed by this enzyme thus other reactions must take place to reduce SeO_3^{2-} to Se^0 and SeH^- . The entry point of selenium into selenocysteine is through *Cys*K of *cys* genes which generates the free amino acid. If this free amino acid gets directly incorporated in to proteins through misacylation of $tRNA^{Cys}$, replacing cysteine would lead to altered activities and functions.

Once inside the cell selenate and selenite are reduced to selenide which gets incorporated into amino acids cysteine and methionine as selenocysteine and selenomethionine. Selenocysteine synthesizes selenomethionine by the same pathway as

methionine, which utilizes cystathione γ-synthase (*met*B), β-cystathionase (*met*C) and methionine synthase (*met*E and *met*H).

Selenite reacts with thiols, especially glutathione which comprises about 90% of total reduced thiol concentration. As it is first in series of reducing selenite to selenide, glutathione is considered the most important component in the selenium metabolism pathway. Reaction of selenite with glutathione leads to the production of hydrogen peroxide (H_2O_2) and superoxide (O_2^-) which indicates the oxidizing ability and toxicity of selenite.[13]

Studies by Muller et al. [99] showed that the selenium gets incorporated into proteins in *E.coli* mutants blocked in the biosynthesis of cysteine. The incorporation required all the enzymatic steps of cysteine biosynthesis except for sulfite reduction suggesting that selenite reduction is a non enzymatic process. The presence of cysteine prevented this non specific incorporation by repressing the cysteine biosynthesis by feedback inhibition. Thus, blocking cysteine biosynthesis as well as incorporation of selenium into proteins which occurs primarily as selenocysteine.[99, 110]

2.6. Glutathione and selenium

Glutathione is suspected to be involved in the selenium metabolism.[71] It is the most abundant thiol in eukaryotic cells, the cyanobacteria and α, β and γ groups of the proteobacteria.

Selenite rapidly reacts in the presence of sulfhydryl groups like glutathione and cysteine and form oxygen radicals, thus it is considered that selenite toxicity in bacteria might be the result of oxidative damage.[71] Work by Kramer et al. [72] suggested that free

radical formation might be involved. Consistently it was observed that two types of superoxide dismutase (SOD) are induced in cultures of *Escherichia coli* exposed to selenite, thus confirming the involvement of free radicals in selenium toxicity. The rate of selenite reduction was observed to decrease when bacteria synthesized lower than normal levels of glutathione. Also, glutathione reductase was induced in cultures of *Rhodobacter sphaeroides* and *E. coli* in the presence of selenite.[18, 73]

Selenite highly reacts with thiol groups forming selenotrisulfides (RS-Se-SR), according to the reaction 1.

$$4\ RSH + H_2SeO_3 \rightarrow RS\text{-}Se\text{-}SR + RSSR + 3H_2O$$

Reaction 1

The selenotrisulfides of glutathione also called as selenodiglutathione, is a very good substrate for glutathione reductase with K_m and V_{max} values comparable with those of glutathione itself. It gets reduced to selenopersulfide of glutathione in the presence of glutathione reductase and NADPH as an electron donor. It follows the reaction 2.

$$GS\text{-}Se\text{-}SG + NADPH \xrightarrow{\text{Glutathione reductase}} GSH + GS\text{-}Se^- + NADP^+$$

Reaction 2

It is also proposed that the unstable selenopersulfide ($GS\text{-}Se^-$) of glutathione dismutates in to elemental selenium (Se^0) and reduced glutathione according to the following stoichiometry (reaction 3).[71, 74]

$$GS\text{-}Se^{-} + H^{+} \rightarrow GSH + Se^{0}$$

Reaction 3

Consequently determination of glutathione level in biological samples has gained interest. Analysis of glutathione can be done by several methods like Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Enzymatic methods, Flow Cytometry and Capillary Electrophoresis. Amongst the varied available methods, HPLC offers sensitivity, selectivity and reproducibility. [76-77, 86-90]

2.7. High Performance Liquid Chromatography (HPLC)

Chromatography is a technique used for separation of a mixture into individual components using a stationary phase and a mobile phase. In liquid chromatography, the components of a mixture are separated by use of a liquid (mobile phase) which carries the mixture across a bed of material (stationary phase). Based on the affinity of the components towards the stationary phase, they travel at different rates across the stationary phase and are eluted out at different times and thus separation is achieved.

The technique of High performance liquid chromatography is so called because of its improved performance, it is also known as High pressure liquid chromatography since high pressure is used when compared to classical column chromatography.[91] High Performance Liquid Chromatography is used in analytical chemistry and biochemistry to identify, quantify and purify the individual components of the mixture. [92] It is widely used for the analysis of pharmaceuticals, biomolecules, polymers and many organic and ionic compounds.[93]

HPLC utilizes different types of stationary phase (saturated carbon chains), pump (solvent delivery system) that moves the mobile phase and analyte through the column, and a detector that provides characteristic retention time for the analyte (the time at which the specific analyte elutes or comes out of the column). Retention time of an analyte varies depending on its affinity towards the stationary phase, the ratio/composition of the solvent(s) used and the flow rate of the mobile phase. The development of smaller particle sizes in HPLC as compared to classical column chromatography provides more surface area which allows for a better separation on columns of shorter length. The pump provides the high pressure required to propel the mobile phase and analyte through the densely packed column as stationary phase. [91, 92]

HPLC is often used as a type of adsorption chromatography with adsorption being the principle of separation. The mixture of compounds (adsorbates) dissolved in the mobile phase travel through the column of stationary phase (adsorbent) according to the relative affinities towards the stationary phase. The compound which has more affinity towards stationary phase travels slowly (thus retention time increased) whereas the compounds with lesser affinity towards the stationary phase travel faster (thus eluted out faster). Hence the compounds are separated. Affinity towards stationary phase is characteristic and typically no two compounds have the same affinity for a combination of stationary phase, mobile phase and other conditions. It also depends upon the mode of chromatography.

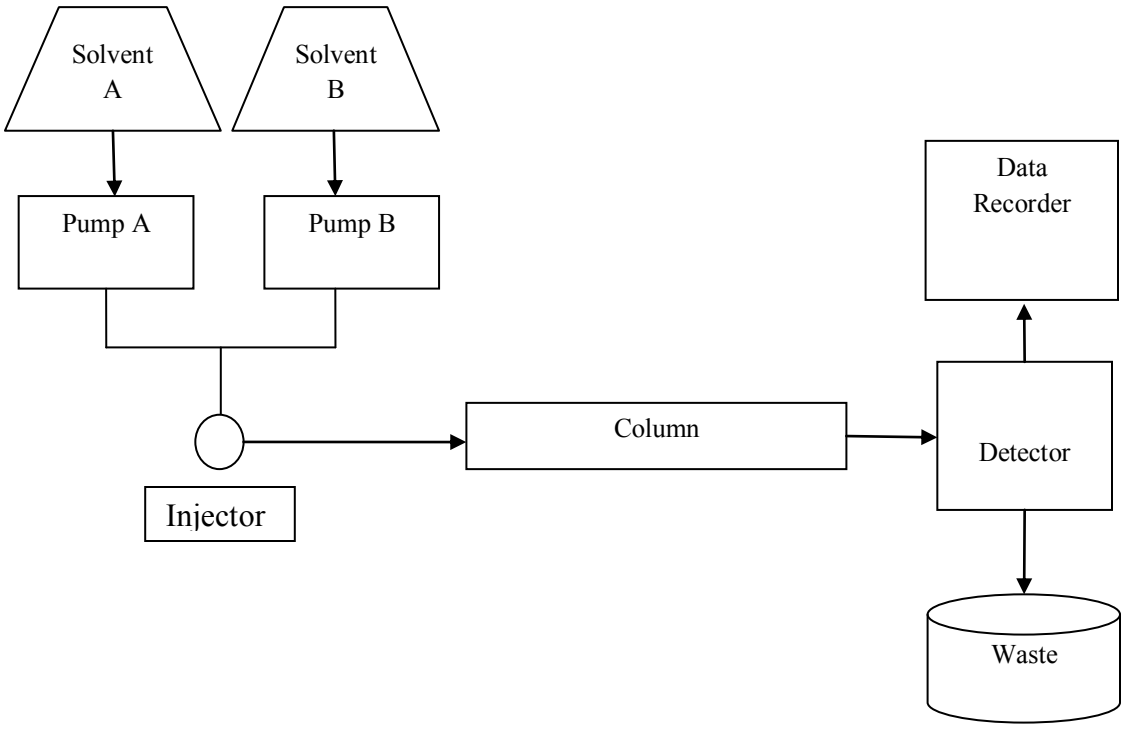


Figure 4. Schematic diagram of HPLC

There are two modes of chromatography – Normal phase mode and Reverse phase mode based upon the polarity of the stationary phase and mobile phase. Polar – polar and non polar – non polar interactions have higher affinity whereas in polar- non polar interaction affinity is less.

Normal phase mode also known as liquid – solid chromatography or adsorption chromatography has polar stationary phase (ex. Silica gel) and non polar mobile phase. Thus, non polar compounds are eluted first because of their lower affinity towards stationary phase. The polar compounds take more time to be eluted from the column as they are retained for a longer time in the column because of more affinity towards the stationary phase. Reverse phase mode has non polar (hydrophobic) stationary phase (ex.

Octadecyl silane (ODS), C_{18}) and polar mobile phase. Hence the polar compounds are eluted first and non polar compounds are retained for a longer time.

The separation also depends upon the elution technique. When same mobile phase combination is used, it is known as isocratic separation, thus maintaining same polarity throughout the process. When a mobile phase combination of lower polarity is used first followed by gradual increase in the polarity it is known as gradient separation.

Reverse phase chromatography is most widely used technique in HPLC and is used in more than 70% of all HPLC analyses [93-95] It is applicable to most non-polar analytes and many ionizable and ionic compounds. As most of the stationary phases in reverse phase technique are hydrophobic in nature, the analytes are separated based on the degree of hydrophobic interaction with the stationary phase.

Figure 5 depicts the view reversed phase chromatography where polar analytes elute out first whereas the non polar analytes interact more strongly with the hydrophobic stationary phase (C_{18} groups) and thus elute out later.[93]

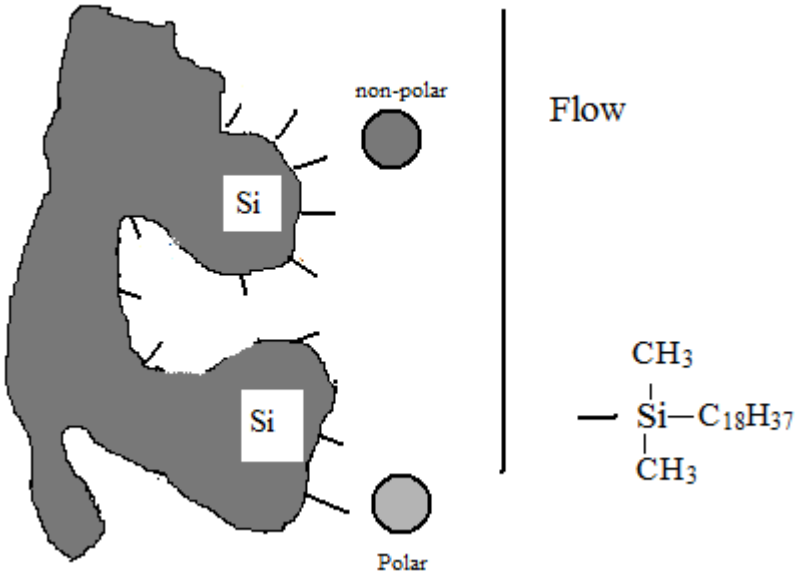
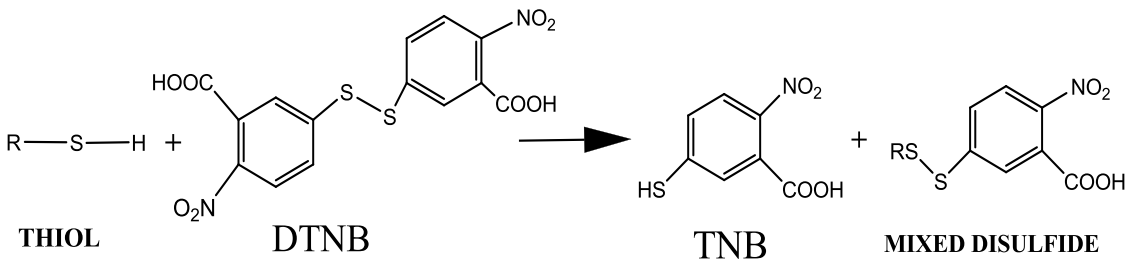


Figure 5. Schematic diagram of part of a porous silica particle with silanol groups (Si-OH) residing at the surface and inside its pores, depicting separation mode of reversed phase chromatography.[93]

2.8. DTNB

Ellman's reagent or DTNB (5, 5' – dithiobis – (2-nitrobenzoic acid) is a symmetric aryl disulfide. It is essentially used to quantify the concentration of thiols present in a sample. Its molecular formula is $C_{14}H_8O_8N_2S_2$. It has a molecular weight of 396.35 g/mol. The reaction with thiols involves the breakage of the disulfide bond on DTNB to release the mixed disulfide (Ellman's derivative) and 2-nitro-5-thiobenzoic acid (yellow colored anion, TNB^-) which is quantified by the absorbance of the dianion (TNB^{2-}) at 412nm. TNB^- ionizes to TNB^{2-} in water at neutral pH or in alkaline pH.



THIOL DTNB TNB **MIXED DISULFIDE**

DTNB is useful in measuring the thiol concentration on proteins and also to measure low-molecular mass thiols such as glutathione.[112] By utilizing a chromatographic method, separation of the disulfide produced is possible after DTNB reacts with reduced thiol.

2.9. Bradford assay

Bradford assay is a rapid and simple assay of protein concentration. It is a colorimetric assay which allows calculation of the total protein concentration (µg or mg) in a sample. Thus, provides total protein quantification.

A colorizing solution called Bradford reagent/ dye consisting of coomassie brilliant blue G-250, which is reddish brown in color (absorbs light 465 nm) reacts with the protein under acidic conditions and is converted into blue form which is absorbs at 595 nm. Thus the protein concentration can be measured by the amount of complex formed in the solution which can be estimated by measuring the absorbance. It is not specific for any particular protein but gives the total concentration of the protein.

2.10. Klett-Summerson photoelectric colorimeter

Colorimetry is defined as the measurement of color and the technique involves evaluation of an unknown color in reference to a known color. It allows determination of the absorbance of a solution at a particular frequency (color) of visual light.

Klett colorimeter is a device used to perform turbidity analyses of a solution, given in Klett units. These Klett units are directly proportional to the optical density or concentration of the solution as per Beer's law. It is not affected by light and temperature. The colorimeter uses specific light filters to obtain the measurements on a Klett scale. The measurements are broadened and also made light and temperature independent by a pair of matched photocells of the "blocking layer" type and a galvanometer of the suspension wire type. The galvanometer with an illuminated logarithmic scale, markings ranging from 1 to 1000 Klett units, provides good accuracy. The scale readings are directly proportional to the concentration of the sample as per Beer's law. Klett can include the blue (400-450 nm), green (520-580 nm) and red glass filter (640-700 nm).

The colorimeter passes a colored light beam through the filter and only one particular color or band of wavelengths of light is transmitted to the photo detector where it is measured. Thus the difference between the amount of light transmitted through the blank and the amount of light transmitted through the sample gives the amount of light absorbed by the sample, which is considered to be directly proportional to the concentration of the sample.

A specifically designed flask with a slanted blind side arm can be used for turbidity measurement in Klett units. Thus, bacterial growth curves can be performed by simple means using Klett colorimeter on multiple volumes of cultures without requiring removal of the cultures for measurements. The optical density can be read at varying ranges. The instrument used had specific light filters with the range from 380 nm-740 nm

To avoid any source of variance or errors, a colorimeter is used to photo electrically measure the light absorbed by a colored sample in reference to a colorless

sample (blank). Klett instrument scale being logarithmic, linear axes is plotted for the growth curves, by plotting time on the x-axis and Klett units (KU) on the y-axis. [91, 102-104]

2.11. Mini Bead beater

A Bead beater is used to disrupt/ lyse the microorganisms. In this technique, the sample solution containing cells or tissues are poured into the bead beater tube containing equal volumes of glass or ceramic beads and is vigorously agitated by shaking or stirring. Thus the disruption takes place by the crushing action of the glass beads when they collide with the cells and amongst themselves. Using this technique, intracellular organelles and membranes can be isolated intact. Disruption of spores, yeast and fungi are most widely done by this technique and has worked successfully with tough to disrupt cells like cyanobacteria, mycobacteria, spores and microalgae.

The size of the beads used is important to the process and 0.1 mm is usually considered for bacteria and spores. Due to gravity, the beads settle down in seconds after the treatment and thus the cell extract can be easily removed. Bead beater (Biospec products) is the most widely used rotor type bead mill which agitates the beads using a rotor.

2.12. Ultra violet –Visible Spectrophotometer (UV - Vis Spectrophotometer)

A Spectrophotometer is an instrument which uses the spectroscopic technique to measure the concentration of a given chemical substance (atomic, molecular or ionic). It is a photometer that measures light intensity as a function of wavelength of light. The absorbance is given in a response plot known as spectrum.

UV - Vis Spectrophotometer is majorly used in physical and analytical chemistry for the identification of substances. It gives the information about electronic transition in

atoms or molecules.[111] It covers the wavelength range from 190-900 nm. An UV-Vis Agilent 8453 series (diode array spectrophotometer) was used for the following experiments. It consists of a tungsten lamp (visible light source) and a deuterium lamp (UV light source). The light beam from the light source is separated into its component wavelengths by the diffraction grating and passes through the sample in a cuvette placed in the sample holder. The intensity of the light is then measured by the detector.

2.12.1. Theory of Spectroscopy

When light is passed through medium containing atoms, molecules or ions, it can be reflected, refracted, scattered, absorbed or transmitted. Spectroscopy is based on the principle that different compounds absorb light at different wavelengths. When the intensity of the emergent light (light coming out) is less than the light passed through (incident light) the sample there is some absorption of energy. The amount of light absorbed is proportional to the concentration of that substance according to Beer's law which is given as:

$$\mathbf{A = \varepsilon b c}$$

where A= absorbance (abs. units)

ε = molar absorptivity (L mol^{-1} cm^{-1})

b = path length (path length of the sample containing cuvette, unit - centimeters)

c = concentration (mol L^{-1})

A sample typically absorbs a band of wavelength from the light source. Only a small difference might be measured between the intensities of light before it passes through the sample versus after it passes through the sample as only small portion of the total amount of light is absorbed by the sample. However if only one wavelength of light is selected to which the sample is sensitive, this difference is increased and the intensity can be measured with ease. The absorbance is represented on a spectrum and is proportional to the amount of sample under study.[91, 111]

Chapter III: Hypothesis

Previous works in the lab resulted in the confirmation that *Enterobacter* sp. YSU is resistant to selenite when grown in M-9 minimal medium in the presence of L-cysteine, while it is sensitive to selenite when grown in the M-9 medium in the absence of L-cysteine. The present research focuses on the selenite toxicity and thiol content of the cells. The hypothesis is that the sensitive cells are killed by reactive oxygen species when the cells are grown in medium containing selenite and lacking cysteine. The presence of cysteine in the growth medium may relieve oxidative stress. Levels of oxidative stress can be determined by measuring the levels of reduced thiols using Ellman's reagent (DTNB) by HPLC and UV - Vis Spectrophotometry. The reaction yields a mixed disulfide (conjugate/complex) and yellow colored TNB (2-nitro 5-thiobenzoic acid). High derivative levels will indicate high reduced thiol levels and low oxidative stress conditions, while low derivative levels will indicate low reduced thiols and high oxidative stress levels.

Chapter IV: Materials and Methods

4.1. Experimental Design

Initially *Enterobacter* sp. YSU cells were aerobically cultured in M-9 minimal media in the presence and absence of L-cysteine. After one and half hours of growth when the cells entered log phase, either selenite or water was added. The cells were collected just before adding the selenite or water, one and half hours after addition, after six hours of growth period and also after twenty four hours of growth. Cell samples were harvested at various time intervals of growth for HPLC analysis - for specific quantification of glutathione (GSH) using DTNB as the derivatizing agent, thiol assay for determination of the total thiol content using 50 mM Tris/HCl pH 8.0, 5 mM EDTA, 0.1% SDS, 0.1 mM DTNB buffer - analyzed by UV - Vis Spectrophotometry and Bradford assay for protein quantification using Bovine Serum Albumin as the standard (BSA). All the cell samples were treated individually.

4.2. Bacterial Strain

The multimetal resistant bacterial strain collected from East fork Poplar creek was believed to be *Stenotrophomonas maltophilia* Oak Ridge strain O2 (S. maltophilia O2) but 16s rDNA sequencing and biochemical tests proved that it was a strain of *Enterobacter*. This new strain was named as *Enterobacter* sp. YSU. *Enterobacter* sp. YSU is a multimetal resistant strain. It has shown resistance to metals like Hg(II), Cd(II), Zn(II), Au(II), Ag(I), As (III) and Se(IV). [99, 100]

4.3. Growth medium

The M-9 minimal medium[105] (Becton, Dickson and Co., Sparks MD, USA) contained 0.24 M disodium phosphate, 0.11 M monopotassium phosphate, 0.04 M

sodium chloride and 0.09 M ammonium chloride, 1 mM magnesium sulfate (Fisher Scientific Fair Lawn, NJ.), 0.2% of glucose (Amresco Inc. Solon, OH) and 0.00005% of thiamine and water, whereas 0.04 mg/mL L-cysteine (Fisher Scientific NJ) and 40 mM sodium selenite (MP Bio Medicals, LLC, Solon, OH) were added as required.

4.4. Growth curve

Two 1000 mL M-9 media were prepared as per table 3, one in the presence and the other in the absence of L-cysteine. Two 10 mL cultures were grown overnight placed in C24 incubator shaker (New Brunswick scientific Co., INC, Edison, NJ) at 37 ^{o}C in the medium prepared and then transferred to 40 mL of corresponding M-9 medium, further grown overnight at 37^{o}C with constant shaking in the incubator at 180 rpm. This starter cultures were transferred to sterile flasks with remaining 950 mL of the respective medium with and without cysteine thus creating 1:20 dilution. In addition, each 1000 mL culture was further divided into half resulting into four flasks each with 500 mL of M-9 medium (two with L-cysteine and two without L-cysteine). All the compounds were aseptically dispensed from stock solutions (either filter sterilized or sterilized by autoclaving). Flasks were capped with sponges in order to maintain aerobic conditions and were incubated at 37^{o}C with constant mixing on a rotary platform shaker. Flasks were respectively labeled as NCNS (No Cysteine, No Selenite), NCS (No Cysteine, Selenite), CNS (Cysteine, No Selenite) and CS (Cysteine, Selenite). Growth was followed by measuring turbidity using Klett colorimeter (Science ware, Belart, Pequamock, NJ) every 45 minutes using 5X M-9 salts as the Blank.

After 90 minutes of growth, or when the growth was reached approximately to 40 Klett units, 15.6 mL of 1 M sodium selenite (Na_2SeO_3, MP Biomedicals, Aurora, OH) was added, taken from a filter sterilized stock solution. Thus 40 mM final selenite concentration was added to the flasks labeled NCS and CS where as 15.6 mL of water was added to NCNS and CNS cultures. 50 mL of each of the four cultures were collected for HPLC analysis at time T0, T1 and T2. 15 mL of each sample was collected for thiol assay and for Bradford assay was collected in triplicates at T0, T1, T2 and T24. Sample (T0), just before adding selenite or water control. Sample (T1), one and half hour after the addition of selenite or water and sample (T2) was collected six hours after the growth. Additional samples (T24), were collected for thiol and Bradford assay after twenty four hours of growth.

4.5. Treatment of samples for reduced thiol (RSH) assay

15 mL of cells collected in triplicates at T0, T1, T2 and T24, centrifuged for 1min at 4^{o}C, 15,000*g to pellet the cells. The cell pellets were frozen and stored at -20^{o}C until further use.

After the frozen pellets were thawed they were resuspended in 1 mL of freshly prepared solution (buffer) of 50 mM Tris/HCl pH 8.0 (Molecular biology grade, Fisher scientific, Fairlawn, NJ), 5 mM EDTA (Biotechnology grade, Solon Ind. Pkwy, Solon, OH), 0.1% SDS (Biotechnology grade, Solon Ind. Pkwy, Solon, OH) and 0.1 mM 5, 5$^{'}$ – dithiobis (2-nitrobenzoic acid) (DTNB) by vortexing (Table 4). Followed by incubating the cell suspensions in an isotemp incubator (Fisher scientific, Fair lawn, NJ) for half

hour at 37^{o}C, briefly vortexed and then centrifuged in a microfuge at 15,000*g for 10 minutes. Supernatant was collected.

A UV – Vis spectrophotometer (Agilent 8453 series) and a cuvette (283 QS 1.000, Fisher Scientific, Fair lawn, NJ) was used to measure the total thiol content. Reagent buffer processed as above was used as the blank. The absorbance of each of the supernatant fraction was measured at 412 nm (wavelength). $1.36X10^{4}$ M^{-1} cm^{-1} was used as the absorption coefficient of oxidized DTNB at this wavelength to calculate the RSH (thiol) concentration of the cell.[106] Concentration was calculated using the Beer-Lambert law.

$$\mathbf{A = \varepsilon b c}$$

Where in

A= Absorbance (abs. units)

ε = molar absorptivity (L mol^{-1} cm^{-1})

b = path length (path length of the sample containing cuvette, unit - centimeters)

c = Concentration (mol L^{-1})

4.6. Treatment of samples collected for HPLC analysis

50 mL of samples collected at T0, T1, T2 were centrifuged (Eppendorf 5810R, Brinkmann instruments Inc, NY) at 7000*g for 7 minutes at 4^{o}C to pellet the cells. Supernatant was discarded and the pellet was resuspended in 1 mL of 1X Phosphate

buffered saline, (PBS) (Amresco, Solon Ind. Pkwy, Solon, OH) of biotechnology grade containing 0.137 M sodium chloride, 0.002 M potassium chloride and 0.01 M phosphate buffer. Then it was transferred in to 1.5 mL sterile microfuge tubes. Centrifuged (Eppendorf 5417R, Brinkmann instruments Inc. NY) again under the same conditions for 7 minutes, repeating the procedure thrice, adding 1 mL PBS each time and discarding the supernatant. The cell pellets were then frozen at -20^{o}C until the termination of the experiment.

The frozen samples were thawed and resuspended by vortexing in 1 mL of 1XM-9 salts. These cell suspensions were poured in to sterile 2 mL Bead beater tubes/vials (Biospec products, Bartlesville, OK), suitable for Mini Bead beater-8 (Biospec products, Bartlesville, OK). The 2.0 mL screw cap with o-ring seals microcentrifuge vials were made up of inert polypropylene which had straight walls and sharp conical bottom. The vials were sterilized by irradiation. These were half filled with 0.1 mm glass beads (Biospec products Inc, Bartlesville, OK). The sample was then lysed by placing it in the Bead beater for 1min, followed by placing the tube in ice for 1 min. The procedure was repeated thrice. Supernatant was collected in new sterile 1.5 mL sterile microfuge tubes and then centrifuged (Eppendorf 5417R, Brinkmann instruments Inc. NY) at 4^{o}C, 15000*g for 5 minutes. Supernatant was collected in new tubes. 10 µL of 0.5 mM DTNB was added to the supernatant, left to react for 10 mins on ice and placed in Standard heat block (VWR scientific products) for 15 mins at above 90^{o}C to denature the proteins. Centrifuged as above for 2 mins, supernatant was collected and froze at -20^{o}C until analysis by HPLC.

4.7. HPLC Analysis

The chromatographic equipment used for HPLC analysis was a Waters 1525 Binary HPLC pump with Waters 2487 Dual λ absorbance detector. A Reversed phase C_{18} column, 250 mm*4.6 mm I.D., 100 Angstrom pore size and 5 µm particle size (Alltech Ultima) was used for separations. Gradient elution was obtained using the mobile phase mixture of Acetonitrile (Fisher Scientific, Fairlawn, NJ) and 140 mM Sodium acetate buffer (Solon Ind. Pkwy, Solon, OH) (pH 5.0, adjusted with acetic acid). Sodium acetate buffer (NaOAC) was used as mobile phase A, it was filtered (Whatman glass fiber paper, 2.5 cm, W&R Balston Ltd.) and Acetonitrile (MeCN) as mobile phase B. The resulting data was analyzed by means of Breeze software on a Dell computer.

L-Glutathione reduced (Sigma-Aldrich, Inc., St. Louis, MO) was used as a standard. A 50 µL sample of serial dilutions of glutathione 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM was each mixed with 50 µL of DTNB, 1 mM, 2 mM, 3 mM, 4 mM and 5 mM respectively. The reaction was allowed to take place for about 10 minutes. The resulting solution was then manually injected in to the HPLC system using a 50 µL loop. The flow rate was maintained at 1 mL/min. The separations were performed at room temperature and the wavelength of detection was 330 nm.

Before running the sample or the standard the column was equilibrated at room temperature with the mobile phase combination of Acetonitrile and degassed NaOAc buffer (adjusted to pH 5.0 by Acetic acid (ACS reagent grade, Pharmco products Inc, Brookfield, CT), at 1 mL/ min for about five to 10 minutes. Then a blank run was carried out with PBS buffer followed by standard and the samples analysis.

4.8. Bead beater

The bead beater used for the present experiments was a Mini bead beater-8 (Biospec products, Bartlesville, OK). It is the first high energy cell disrupter which can handle multiple vial samples (eight, 1.5 or 2.0 mL each) at one time. The 2.0 mL micro centrifuge vials (Bio spec products, Bartlesville, OK) used were made up of polypropylene and were sterilized by irradiation. They are 2.0 mL screw capped with o-ring seals having straight walls with a sharp conical bottom. Maximum efficiency is provided due to top to bottom near horizontal shaking with about 100-2800 oscillations/min (variable speed). One to eight standard bead beater tubes containing ceramic or glass beads and microbial cells or plant and animal tissues can be disrupted by violent agitation. It completely homogenizes resistant samples like yeast, spores or fibrous tissue in about 3 minutes in 0.1 to 1 mL of extraction medium. Enzymes and organelles are preserved due to non foaming, aerosol free method. [109]

4.9. Treatment of samples for Bradford assay

Additional samples were removed at T0, T1, T2, and T24 for the determination of cell protein content. This allowed for the RSH concentration to be normalized to total cell protein.[4] 15 mL of cells collected at T0, T1, T2 and T24 were centrifuged for 1 min at 4^{o}C, 15,000*g to pellet the cells. The cell pellets were frozen and stored at -20^{o}C until further use.

The frozen samples were thawed and resuspended by vortexing in 1 mL of 1X PBS (Phosphate buffered saline) buffer. These cell suspensions were poured in to sterile 2 mL bead beater tubes, which was half filled with 0.1 mM glass beads (1 mL). The

sample was then lysed by placing it in the Bead beater for 1 min, followed by placing the tube in ice for a min. the procedure was repeated thrice. Cleared lysates were generated by centrifugation at 15000*g for 5 minutes at 4^{o}C. Supernatant was collected in new sterile 1.5 mL microfuge tubes and the protein content of each lysate was determined by using the method of Bradford.

UV - Vis Spectrophotometer (Agilent 8453 series) was used to measure the total protein concentration a Cuvette (284 QS 10.00 mm, Fisher scientific). Bradford assay was performed using the Bradford dye. Bovine Serum Albumin, BSA (2 mg/mL) (Pierce, Rockford, IL) prepared in 0.9% of aqueous NaCl solution containing sodium azide was used as the standard. BSA (1 mg/mL) was prepared from the stock by diluting with 1X PBS. Bradford dye and PBS (1X) were used as blank. The absorbance of each of fraction was measured at 595 nm.

4.9.1. Calibration curve

Calibration curve was obtained using BSA (1 mg/mL) as the protein standard. Bradford dye/reagent mixed with 1X PBS was used as blank. A series of test tubes with varying amounts of BSA and a constant amount of Bradford reagent was set up first Bradford reagent was placed in the test tube (13*100 mm borosilicate glass, VMR international, West Chester, PA) followed by PBS, then appropriate amount of standard protein was added, mixed and incubated for 10 minutes. The absorbance was measured at 595 nm using UV - Vis Spectrophotometer. Amount of components added were followed as in table 1. Same procedure was followed for the sample which was added instead of the standard BSA (Table 2). Protein concentration in the sample was estimated in

reference to the absorbance obtained from a series of standard protein dilutions (calibration curve). The calibration curve was drawn by plotting absorbance data on the y-axis whereas the amount of BSA (µg) on the x-axis.[107, 108]

The equation of line obtained by the standard curve was used to estimate protein concentration

$$\mathbf{y=mx+c}$$

Where m = slope

c = x-intercept

x = concentration

y = absorbance

Table 1. Bradford assay standard concentration table

TEST TUBE #	SAMPLE	BRADFORD DYE (mL)	BSA (1mg/mL) µL	PBS (1X) µL
B	BLANK	3	0	100
S1	STANDARD 1	3	10	90
S2	STANDARD 2	3	30	70
S3	STANDARD 3	3	50	50
S4	STANDARD 4	3	70	30
S5	STANDARD 5	3	90	10

Table 2. Bradford assay sample data table

S.No.	SAMPLE NAME	COOMASSIE REAGENT (mL)	PBS (1X) (µL)	AMOUNT OF SAMPLE (µL)
1	NCNS T0	3	0	100
2	NCS T0	3	0	100
3	CNS T0	3	0	100
4	CS T0	3	0	100
5	NCNS T1	3	50	50
6	NCS T1	3	0	100
7	CNS T1	3	50	50
8	CS T1	3	0	100
9	NCNS T2	3	80	20
10	NCS T2	3	0	100
11	CNS T2	3	80	20
12	CS T2	3	50	50

Note – Sterile microfuge tubes, bead beater tubes and pipet tips were used throughout. All compounds were aseptically dispensed from filter (autoclave) sterilized stock solutions.

Table 3. Preparation of 1000 mL of M-9 minimal media

COMPONENTS	VOLUME NEEDED (500 mL WITH CYSTEINE) in mL	VOLUME NEEDED (500 mL NO CYSTEINE) in mL
5X M-9 salts	200	200
20% Glucose	10	10
1 M $MgSO_4$	1	1
0.5% Thiamine	0.1	0.1
Cysteine (4 mg/mL)	10	-
Water (sterile)	Make up to 1000 mL	Make up to 1000 mL

Table 4. Preparation of thiol assay buffer (60 mL)

Components	**Volume (mL)**
1 M Tris/HCl pH 8.0	3
0.5 M EDTA	0.06
20% SDS	0.3
5 mM DTNB	1.2
Water	55.44

4.10. Preparation of 100 mL of 1X PBS (Phosphate buffered saline)

By dissolving one tablet of PBS (Amresco, Solon Ind. Pkwy, Solon, OH) in 100 mL of deionized water 10X of solution was prepared. Autoclaved (HIclave HVE-50, Amerex instruments, Inc, Lafayette, CA) at 121^{o}C for 15 minutes for sterility.

4.11. Preparation of 1 L of 5X M-9 salts

Dissolved 56.4 g of M-9 salts in 1000 mL of deionized water to prepare 1 L of 5X M-9 salts, followed by autoclaving at 121^{o}C for 15 minutes.

4.12. Preparation of 1 L of Bradford Dye/Reagent

100 mg of Brilliant blue G-250 (Fisher scientific, Fairlawn, NJ) of electrophoresis grade was dissolved in 50 mL of 95% ethanol (Acros, NJ), spectrophotometric grade, in a 1000 mL beaker. Followed by, addition of 100 mL of 85% w/v Phosphoric acid (Fisher scientific, Fair Lawn, NJ). Diluted to 1 L using Milli Q water and filtered thoroughly using a whatman filter paper (100 circle, 32.0 cm, W & R Balston limited)

CHAPTER V: RESULTS

The role of cysteine in selenite resistance was confirmed by performing growth curves on *Enterobacter* sp. YSU strain. Four overnight cultures grown in M-9 minimal medium were diluted in 1: 20 M-9 medium. Two cultures contained cysteine and two cultures lacked cysteine. After 1.5 hours of growth at 37^{o}C, selenite was added to final concentration of 40 mM to NCS and CS. In NCNS and CNS equal volumes of water was added as a control. Thus, four conditions were maintained, NCNS (No Cysteine, No Selenite), NCS (No Cysteine Selenite), CNS (Cysteine, No Selenite) and CS (Cysteine, Selenite). Optical density was measured after every 45 minutes using a Klett Colorimeter. The average turbidity of NCNS, CNS and CS after about six hours of growth was 206, 189 and 306 Klett units whereas the turbidity of NCS was 63 Klett units. NCNS, CNS and CS showed exponential growth where as NCS failed to grow after the addition of selenite. Thus, the bacterial strain seemed to be resistant to 40 mM selenite in the presence of L-cysteine.

Figure 6 represents typical growth curve of bacteria involving four stages: Lag, log, stationary and death phase. During the lag phase bacteria starts to acclimatize in the new environment. There is an exponential growth in the log phase, during this phase the metal of interest is added in to the culture. At stationary phase it runs out of nutrition and thus the growth stops finally leading to the death phase. [113]

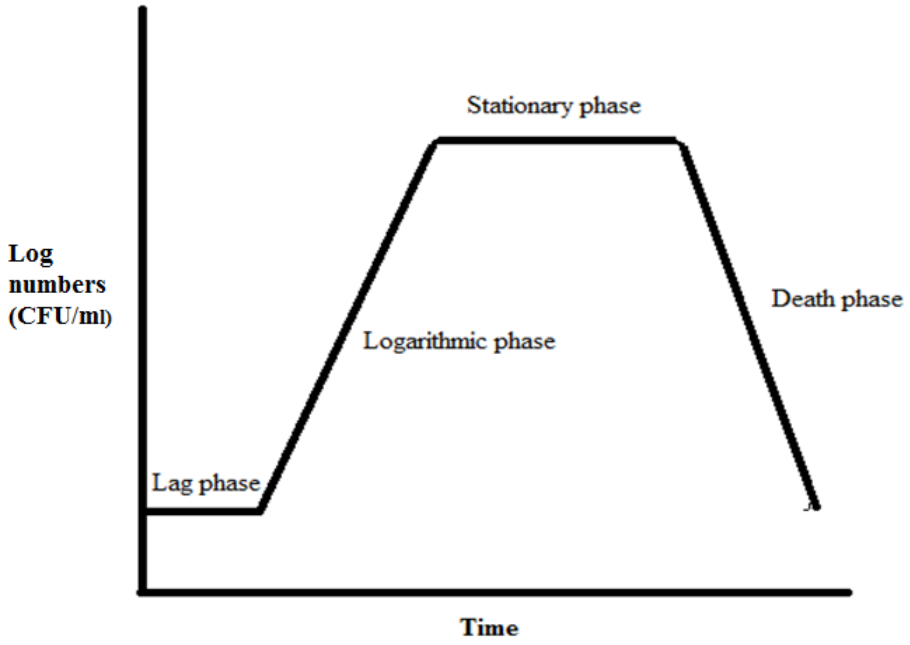


Figure 6. Microbial growth curve (Lag, log, stationary, death Phase)

5.1. Growth curves of *Enterobacter* sp. YSU

Turbidity was measured using a Klett colorimeter after every 45 minutes of growth for six hours. Table 5 shows average measurements for nine individual growth curves. The samples in the presence of selenite and no cysteine lacked growth after the addition of selenite whereas the samples which had selenite and cysteine showed exponential growth along with the samples used as control. Thus cysteine appears to help in cell growth in the presence of selenite. In the following table, NCNS = No Cysteine, No Selenite, NCS = No Cysteine, Selenite, CNS = Cysteine, No Selenite, CS = Cysteine, Selenite.

Table 5. Influence of cysteine on selenite resistance in *Enterobacter* sp. YSU

TIME (min)	NCNS	NCS	CNS	CS
	Klett units			
0	19	21	21	21
45	23	25	25	25
90	38	38	41	40
135	57	47	64	62
180	93	55	96	94
225	133	59	136	130
270	176	61	175	177
315	204	62	191	243
360	206	63	189	306

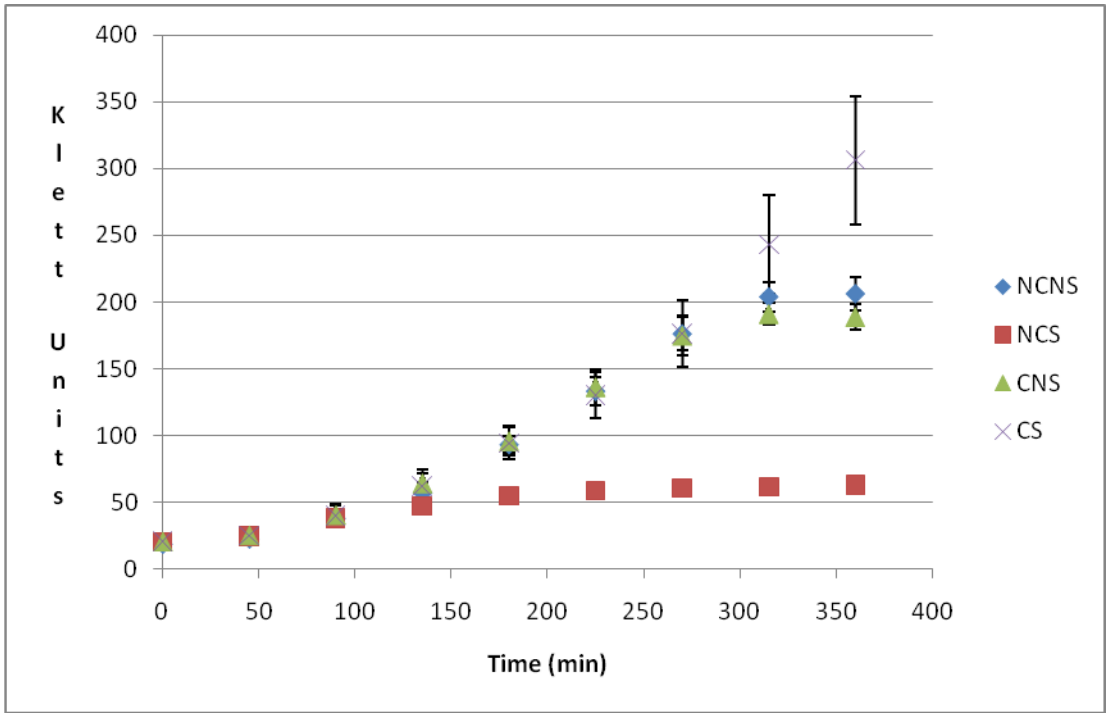
NCNS = No Cysteine, No Selenite

NCS = No Cysteine, Selenite

CNS = Cysteine, No Selenite

CS = Cysteine, Selenite

Figure 7. *Enterobacter* sp. YSU growth curve. It shows time in minutes versus Klett Unit measurements of *Enterobacter* YSU growth curve. Samples were grown in fresh M-9 medium in the presence and absence of cysteine and selenite. After 1.5 hours of growth 40 mM selenite or equal volumes of water was added to each culture. The values at each point represent the average values of nine individual growth curves. Student t test at 95% confidence level was used to calculate the error. In the following plot, NCNS = No Cysteine, No Selenite, NCS = No Cysteine, Selenite, CNS = Cysteine, No Selenite, CS = Cysteine, Selenite


Klett Units
400
350
300
250
200
150
100
50
0
0
50
100
150
200
250
300
350
400
Time (min)
NCNS
NCS
CNS
CS

.

5.2. Bradford results

Bradford assays were performed to calculate the total protein concentration in the cultures. It is a colorimetric assay which gives the total protein concentration. It is not a specific technique for a particular protein but the total protein concentration. The Bradford reagent (Coomassie brilliant blue) reacts with the protein in the sample in an acidic solution resulting in the absorbance shift from 465 nm to 595 nm. The color of the reagent changes from brownish red to blue. Bovine Serum albumin was used as the standard. 15 mL samples were collected three times: just before adding selenite (T0), one and half hour after addition of selenite (T1) and after six hours of growth (T2). Various amounts of the standard were mixed with a constant amount of the Bradford reagent and the absorbance was measured using UV - Vis Spectrophotometry at 595 nm as shown in table 6. A calibration curve was plotted by plotting the average absorbance of triplicate trials on the y-axis and amount of the standard (µg) on the x –axis, which yielded an R^2 value of 0.9838. Representative data are shown for one of the growth curves in figure 8. The equation of the line Y=mX+C obtained was used to determine the total protein concentrations in the samples. Y is the average absorbance which is substituted in the formula and solved for the desired concentration in µg. The number was then divided by the amount of micro liters of sample mixed with the reagent to get the concentration of protein in or micrograms per microliter, milligrams per milliliter. To get the concentration in milligram per milliliter the number was divided by 15 (as the samples were 15 times concentrated). Thus, the concentration of sample was obtained in mg/mL.

A graph was plotted between time in hours (considering the time of sample collection) on the x-axis and the averaged total protein concentration (mg/mL) in the

samples on the y-axis figure 9. It was observed that the protein content in NCNS, CNS and CS increased where as the total protein concentration of NCS declined. Thus, it appears that selenite is toxic to the *Enterobacter* sp. YSU, but is resistant when cysteine is added in the culture medium. Thus, cysteine seems to help in providing resistance and also that the total protein concentration is effected in the presence of selenite.

Table 6 represents the average absorbance data of BSA (µg) of a single growth curve where measurements were taken in triplicate.

Table 6. Bradford assay standard absorbance data

	ABSORBANCE at 595 nm			
BSA (μg)	TRIAL 1	TRIAL 2	TRIAL 3	AVERAGE
10	0.1644	0.1470	0.2905	0.2006
30	0.4620	0.4016	0.4352	0.4330
50	0.6551	0.6023	0.6966	0.6513
70	0.8950	0.8074	0.9742	0.8922
90	1.017	0.9667	1.041	1.008

Figure 8. Bradford assay calibration curve. The calibration curve was obtained using BSA (Bovine Albumin Serum, 1 mg/mL) as the standard. Serial dilutions of the standard were mixed with Bradford dye as per table 1 and the absorbance was measured at 595 nm. The absorbance was plotted on the y –axis and the amount of BSA (μg) on the x-axis. The data are fit to a linear curve with an R^2 value of 0.9838. The equation of line y=0.0112x+0.0649 was used to determine the total protein concentrations in the sample.

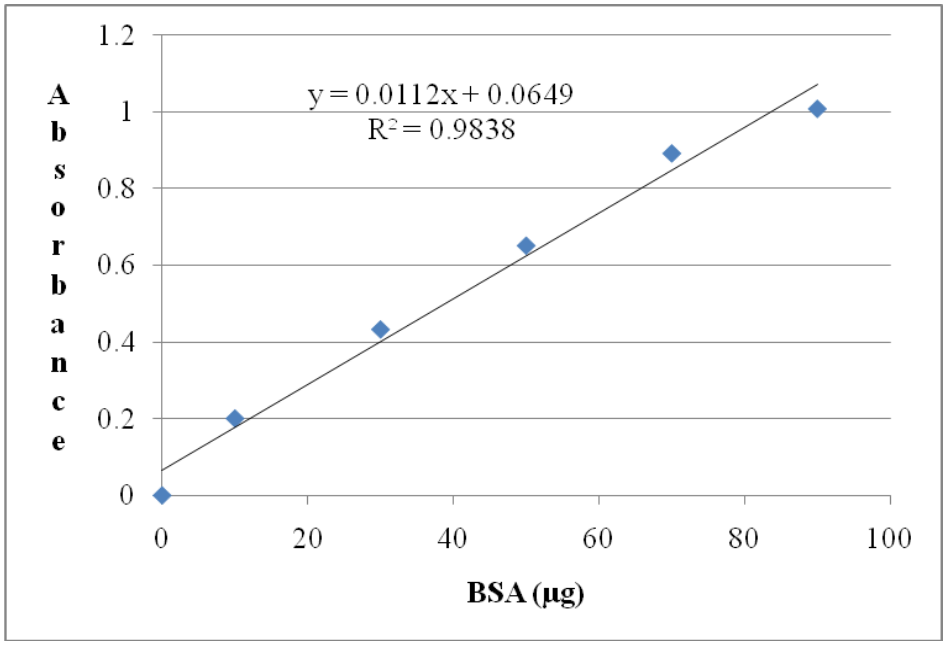
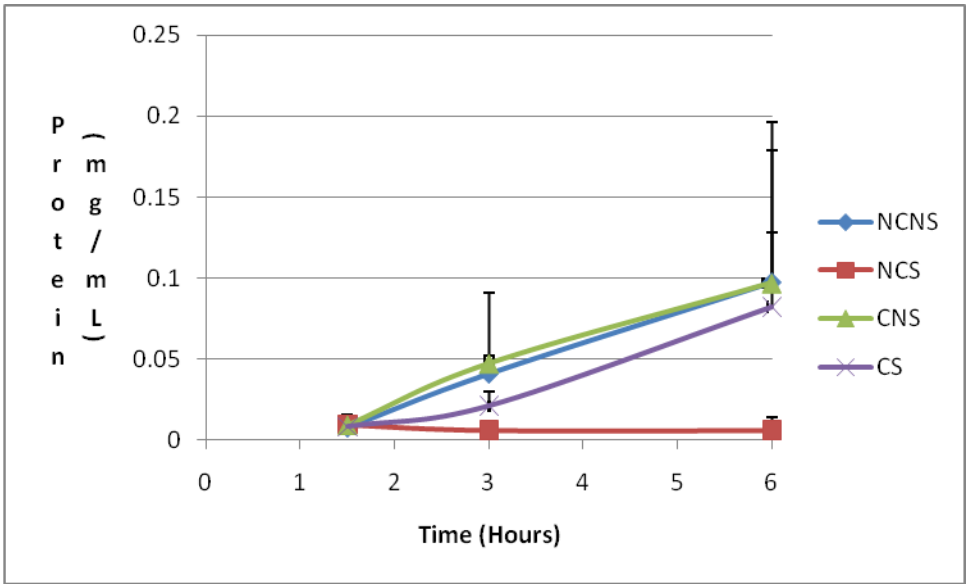

y = 0.0112x + 0.0649
R² = 0.9838
Absorbance
BSA (µg)
0
0.2
0.4
0.6
0.8
1
1.2
0
20
40
60
80
100

Table 7 Average total protein concentration data of samples. The absorbance of individual growth curves measured in triplicates using UV – Vis Spectrophotometer at 595 nm were averaged. The y=mx+c value obtained from the calibration curve was used to calculate the mass of protein (µg). The value was divided by the amount of sample (µL) mixed with the reagent and then divided by 15 (as the samples were 15 times concentrated). Thus, the total protein concentration in (mg/mL) was obtained. The average concentration value in the table represents the average concentration of the total protein (mg/mL) obtained from three individual growth curves.

Table 7. Averaged total protein concentration data of samples

SAMPLE	AVERAGE CONCENTRATION OF PROTEIN (mg/mL)
NCNS T0	0.007432
NCS T0	0.009545
CNS T0	0.009419
CS T0	0.008199
NCNS T1	0.04068
NCS T1	0.006104
CNS T1	0.04736
CS T1	0.02097
NCNS T2	0.09699
NCS T2	0.005977
CNS T2	0.09687
CS T2	0.08207

Figure 9. Effect of selenite on total protein concentration. It represents the average total protein concentration of the samples where time in hours is plotted on the x-axis and concentration in (mg/mL) on the y-axis. The amount of sample taken is tabulated in table 2 and the absorbance was measured at 595 nm. The equation of line was used to calculate the total protein concentration in mg/mL. The graph showed increase in total protein concentrations of NCNS, CNS and CS where as the total protein concentration in NCS declined. The figure represents average value of three growth curves whose absorbance was measured in triplicates each. Error was calculated by student t test at 95% confidence level. Thus, the figure shows the role of cysteine in selenite resistance and the toxic effects of Selenite resulting in the decrease of total protein concentration. Where NCNS = No Cysteine, No Selenite, NCS = No Cysteine, Selenite, CNS = Cysteine, No Selenite, CS = Cysteine, Selenite


0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
6
Time (Hours)
Protein (mg/mL)
NCNS
NCS
CNS
CS

5.3. Thiol assay result

Growth curves were performed on *Enterobacter* sp. YSU cells in the presence of selenite (40 mM). Samples were collected at different time periods for thiol assay. The thiol concentration was measured by treating the samples with a buffer containing Ellman's reagent (DTNB), which effectively quantifies the reduced thiol content in samples. The buffer also had EDTA and SDS which provided cell lysis and protein denaturing conditions. As such, all the cysteines could be available for the reagent. Thus the reduced thiol content was measured by checking absorbance at 412 nm using a UV - Vis Spectrophotometer.

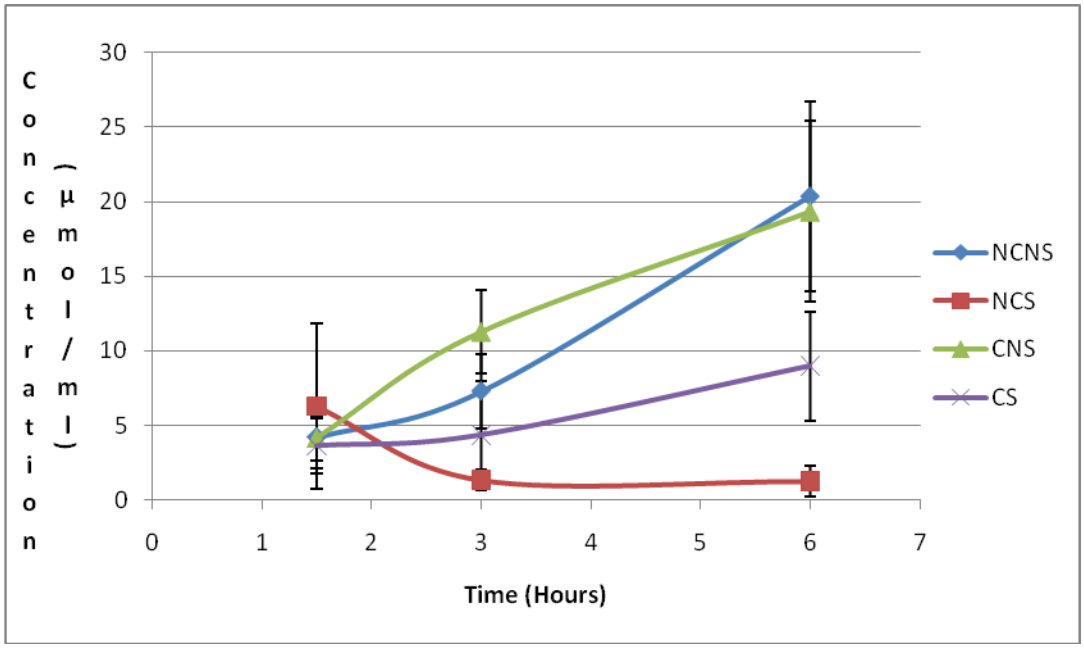
It was assumed that reactive oxygen species are evolved when cells are grown in the medium containing selenite and lacking cysteine. This was considered to be the reason for oxidative stress which was assumed to be relieved by the presence of cysteine in the growth medium.

The data provided shows an average concentration (µmol/mL) value of seven individual growth curves (Table 8). Following Beer's law, the concentration in micromoles was calculated using the absorption coefficient of oxidized DTNB ($1.36 * 10^4$ $M^{-1}cm^{-1}$). A graph was plotted with time in hours on the x-axis and the concentration (µmol/mL) on the y-axis. Cell cultures exposed to selenite and lacking cysteine showed loss of cellular thiol content compared to the cultures grown in the presence of with or without selenite and cysteine.

Table 8. Average thiol concentration data

Sample	Concentration (µmol/mL)
NCNS T0	4.214
NCS T0	6.281
CNS T0	4.164
CS T0	3.639
NCNS T1	7.289
NCS T1	1.331
CNS T1	11.27
CS T1	4.364
NCNS T2	20.37
NCS T2	1.248
CNS T2	19.34
CS T2	8.975

Figure 10. Effect of selenite on thiol concentration. It represents the thiol concentration (µmol/mL) plotted on the y axis as per time in hours on the x-axis. The values at each point are the average values of seven individual growth curves. The samples collected at different time periods were treated with buffer containing DTNB which quantifies the thiols present effectively. Where NCNS = No Cysteine, No Selenite, NCS = No Cysteine, Selenite, CNS = Cysteine, No Selenite, CS = Cysteine, Selenite.


Concentration (µmol/ml)
30
25
20
15
10
5
0
0
1
2
3
4
5
6
7
Time (Hours)
NCNS
NCS
CNS
CS

5.4. Normalized reduced thiol content per total cell protein

The effect of selenite exposure on the total reduced thiol content of *Enterobacter* sp. YSU was analyzed. Thus, to calculate the total reduced thiol content (RSH) per milligram of cell protein, the thiol concentrations (μmol/mL) were divided by the total protein content (mg/mL). The thiol concentrations were obtained using a buffer containing DTNB and measuring the absorbance at 412 nm, while the total protein content was measured using Bradford dye and BSA as the standard. The calibration curve from the standard gave the equation of the line which was used to calculate the total protein concentration in the samples.

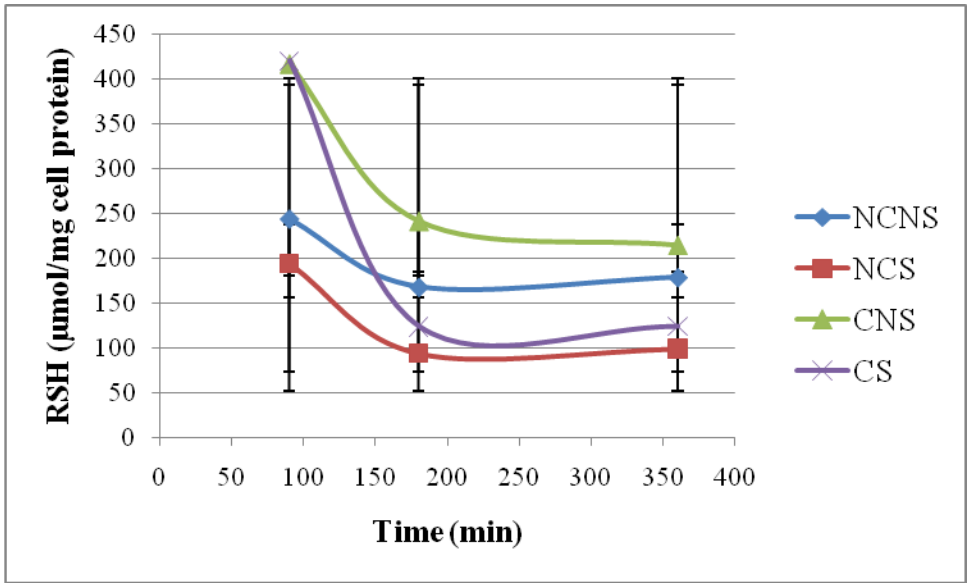
15 mL of cells were collected for total thiol and protein assay at different time points. All the data provided is an average of two individual growth curves measured in triplicate. NCNS, CNS showed much stability in the level of reduced thiol content per cell, while NCS showed loss of RSH concurrently with the loss of culture viability. In NCS, thiol oxidation (as seen by the reduced thiol content) occurred immediately after the addition of selenite until it stabilized. Not much difference was observed between the thiol oxidation response of NCS and CS. While CS showed some amount of resistance to selenite as seen by the increased thiol concentration and protein concentration (Figure 11), total RSH level per cell response was not maintained as compared to the standard.

Table 9 represents the normalized thiol content in μmol/mg cell protein values of the sample averaged from two individual growth curves.

Table 9. Normalized thiol content data per milligram cell protein

Sample	Thiol concentration (µmol/mg cell protein)
NCNS T0	244.0
NCS T0	194.5
CNS T0	417.7
CS T0	420.8
NCNS T1	168.2
NCS T1	94.40
CNS T1	241.3
CS T1	124.1
NCNS T2	179.0
NCS T2	99.54
CNS T2	214.4
CS T2	124.2

Figure 11. Normalized thiol content per cell protein. It plots time in minutes on the x-axis and RSH content (µmol /mg cell protein) on the y-axis. The reduced thiol content per cell was calculated by dividing the total thiol concentration (µmol) with total protein concentration (mg/L) obtained from the Bradford assay. The values represent the average data of two individual growth curves. Where NCNS = No Cysteine, No Selenite, NCS = No Cysteine, Selenite, CNS = Cysteine, No Selenite, CS = Cysteine, Selenite.


RSH (µmol/mg cell protein)
450
400
350
300
250
200
150
100
50
0
0
50
100
150
200
250
300
350
400
Time (min)
NCNS
NCS
CNS
CS

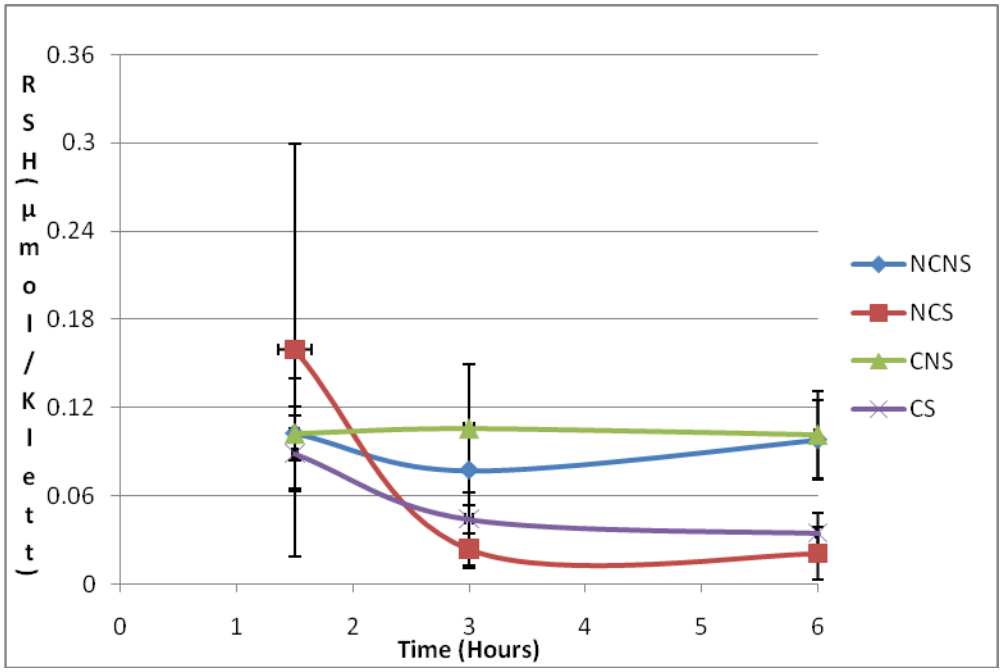
5.5. Normalized reduced thiol content per Klett unit

Suspecting the need for more Bradford assay trials for calculating the normalized thiol concentration per cell, the normalized thiol per Klett unit was calculated using the Klett data. It was calculated by dividing total thiol content obtained by using the molar absorption coefficient value of oxidized DTNB ($1.36* 10^4$ $M^{-1}cm^{-1}$) with that of the Klett units obtained at T0, T1 and T2. T0 represents the samples collected just before adding selenite or water control. T1 represents the samples collected one and half after the addition of selenite or water control and T2 represents the samples collected after six hours of growth. Data presented in (table 10) is the average RSH value of seven individual growth curves.

Table 10. Normalized thiol concentration per Klett

Sample	Average RSH (µmol/Klett units)
NCNS T0	0.1027
NCS T0	0.1593
CNS T0	0.1025
CS T0	0.08874
NCNS T1	0.07737
NCS T1	0.02383
CNS T1	0.1058
CS T1	0.04420
NCNS T2	0.09822
NCS T2	0.02095
CNS T2	0.1017
CS T2	0.03475

Figure 12. Normalized thiol content per Klett. It presents time in hours on the x-axis and thiol concentration divided by Klett Units on the y- axis. The thiol concentrations of samples collected at T0, T1 and T2 were measured using UV - Vis Spectrophotometer at 412nm in the presence of DTNB. The amount of thiol is divided by the respective Klett units measured at T0, T1 and T2. The values at each point represent average of seven individual growth curves. A student t test with 95% confidence was used to calculate error. Where NCNS = No Cysteine, No Selenite, NCS = No Cysteine, Selenite, CNS = Cysteine, No Selenite, CS = Cysteine, Selenite.

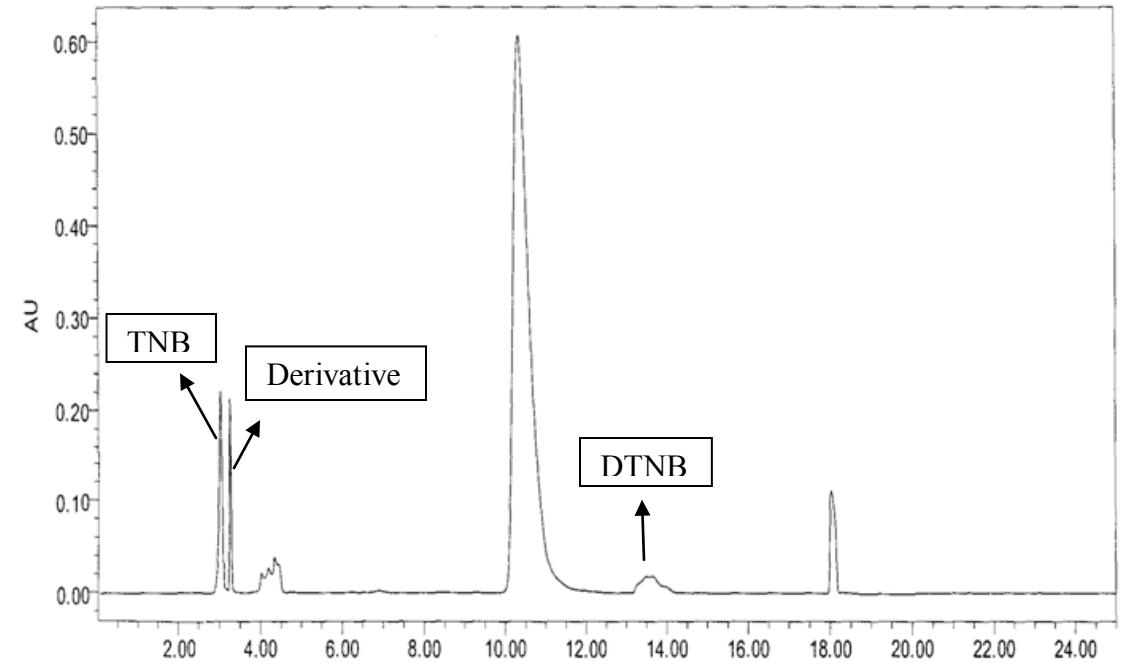

0.36
0.3
0.24
0.18
0.12
0.06
0
RSH (µmol/Klet)
0
1
2
3
4
5
6
Time (Hours)
NCNS
NCS
CNS
CS

5.6. HPLC analysis

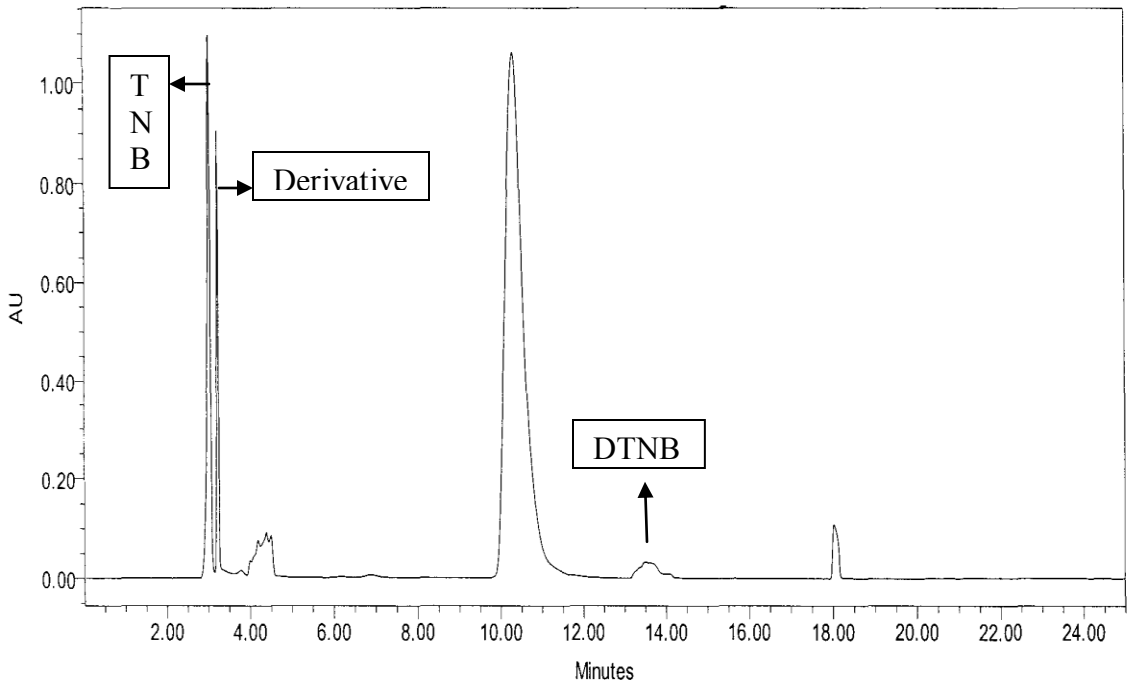
HPLC analysis was done using Waters 1525 Binary HPLC pump connected to Waters 2487 Dual λ absorbance detector. The samples were injected using a 50 µL loop in to the HPLC system which contained a Reversed phase C_{18} column as the stationary phase and the mobile phase was a mixture of 140 mM NaOAc buffer (pH 5.0) and acetonitrile. The flow rate was maintained at 1 mL/min with run time of 25 minutes. HPLC analysis was performed at 330 nm.

The column was equilibrated with mobile phase and a blank with PBS buffer was run. L-glutathione reduced was used as a standard. The samples and the standard were reacted with excess of Ellman's reagent (DTNB) which results in the formation of a derivative and TNB. In order to obtain reliable determinations, samples containing only standard (No DTNB) and only DTNB were also assayed to confirm the retention time of DTNB and RSH and also the conjugate formed.

The GSH standard solutions were analyzed at 330 nm, after derivatization with two fold excess DTNB, to allow all the thiols present to react with the reagent. The experiments were performed at room temperature. HPLC analysis of GSH standard's of 0.5 mM and 1.0 mM is shown in figure 13. The derivative was eluted at around 3.3 min. While the unreacted DTNB eluted at around 14.0 min, the TNB (Ellman monomer) had a retention time of 3.0 min. The retention time of DTNB was confirmed by running only 5 mM of DTNB which eluted at around 14.0 min. The derivative peak increased at 3.3 as the concentration of GSH was increased in the standard thus giving a linear curve.

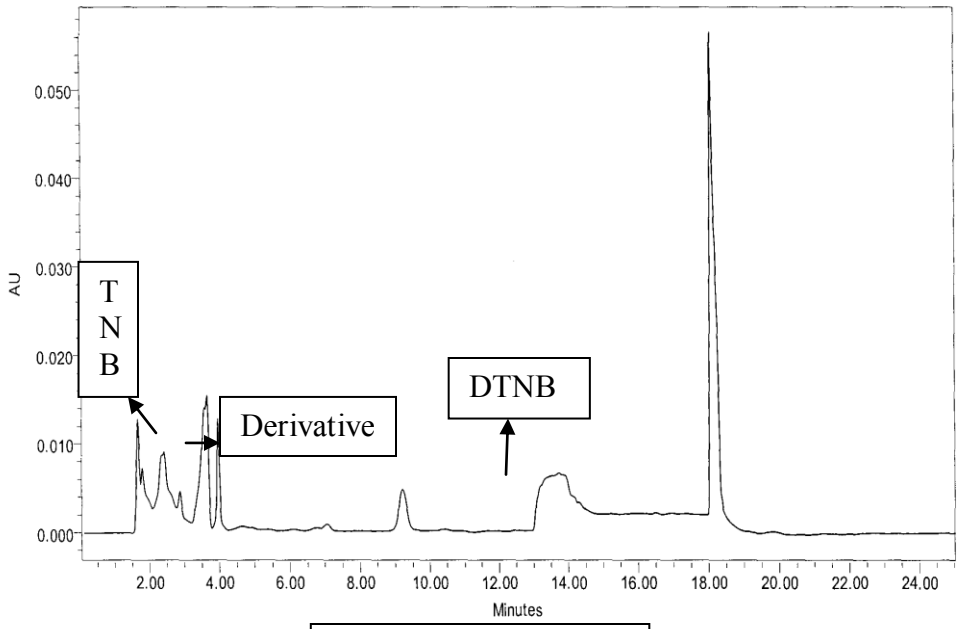


0.5 mM Glutathione

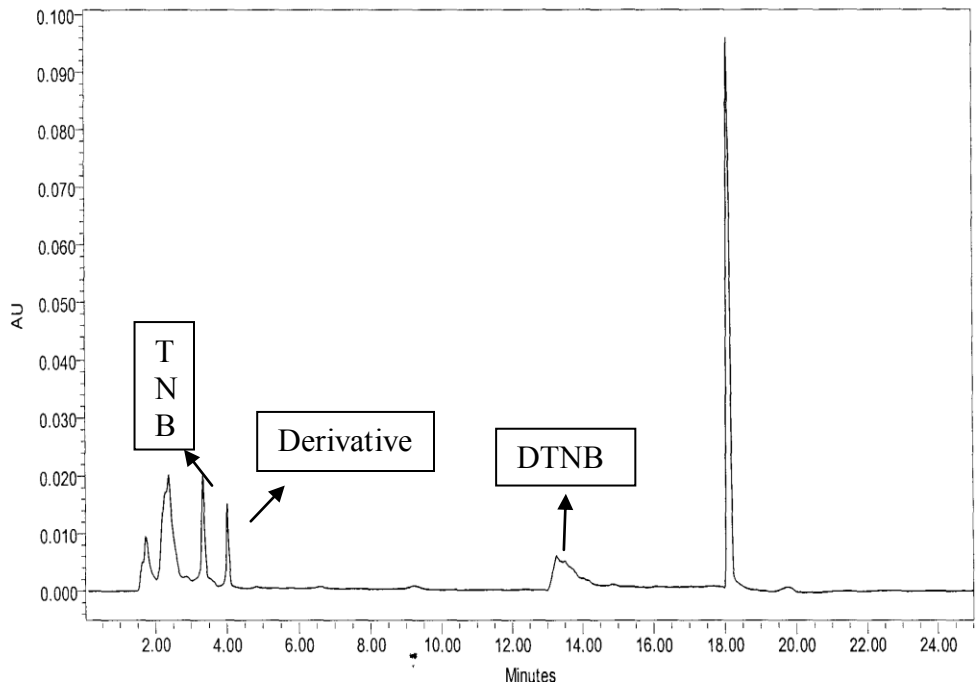


1.0 mM Glutathione

Figure 14 represents the CS T2 (Cysteine, Selenite) chromatogram and CNS T2 (Cysteine, No Selenite) chromatogram of an individual growth curve. Similar to the glutathione standard results, the derivative eluted at around 3.4 minutes. The TNB showed a retention time around 3.1 minutes. While the unreacted DTNB eluted at around 14.0 minutes in the samples as well.



CS T2 Chromatogram



CNS T2 Chromatogram

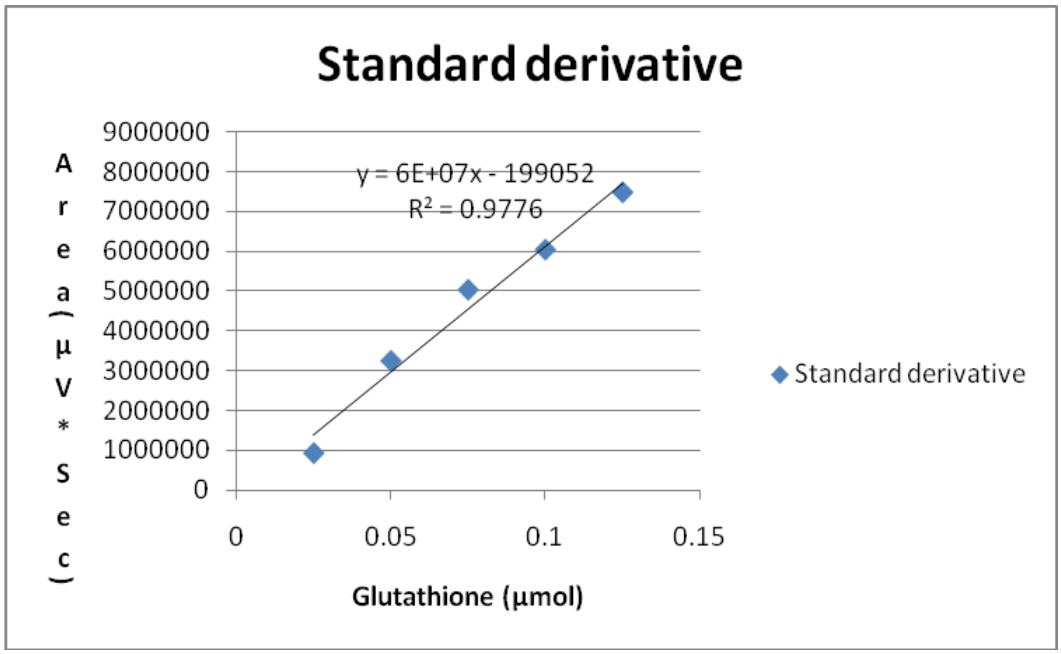
L-Glutathione reduced was used as a standard. A 50 µL sample of serial dilutions of glutathione 0.5 mM, 1.0 mM, 1.5 mM, 2.0 mM, 2.5 mM were each mixed with 50 µL of DTNB, 1 mM, 2 mM, 3 mM, 4 mM and 5 mM respectively. The reaction was allowed to take place for about 10 minutes. The resulting solution was then manually injected in to the HPLC system using a 50 µL loop.

Table 11 represents the average area (µV*Sec) of the peak data of glutathione derivative formed when reacted with DTNB. Area of the peak was calculated by integration. The peak area presented is the average value of measurements taken in triplicate, which is tabulated with the micromole amounts of glutathione. By plotting the area under the peak (µV*Sec) value on the y-axis and the µmol amounts of the glutathione standard on the x axis, a calibration curve was obtained. (figure 15)

Table 11. Average peak area data of standard glutathione derivative

GSH derivative (µmol)	Average peak area (µV*Sec)
0.025	949673.3
0.05	3253687
0.075	5032998
0.1	6038012
0.125	7471082

Figure 15 HPLC calibration curve. It represents the calibration curve obtained by plotting area (μV*Sec) of the derivative peak on the y –axis which was obtained when glutathione was reacted with DTNB. The glutathione eluted at 3.3 minutes and the amounts (μmol) were plotted on the x-axis. It resulted in a correlation coefficient of 0.9776 and y=6E+07x-199052 was the equation of the line.


Standard derivative
Area (µV*Sec)
9000000
8000000
7000000
6000000
5000000
4000000
3000000
2000000
1000000
0
y = 6E+07x - 199052
R² = 0.9776
0
0.05
0.1
0.15
Glutathione (µmol)
Standard derivative

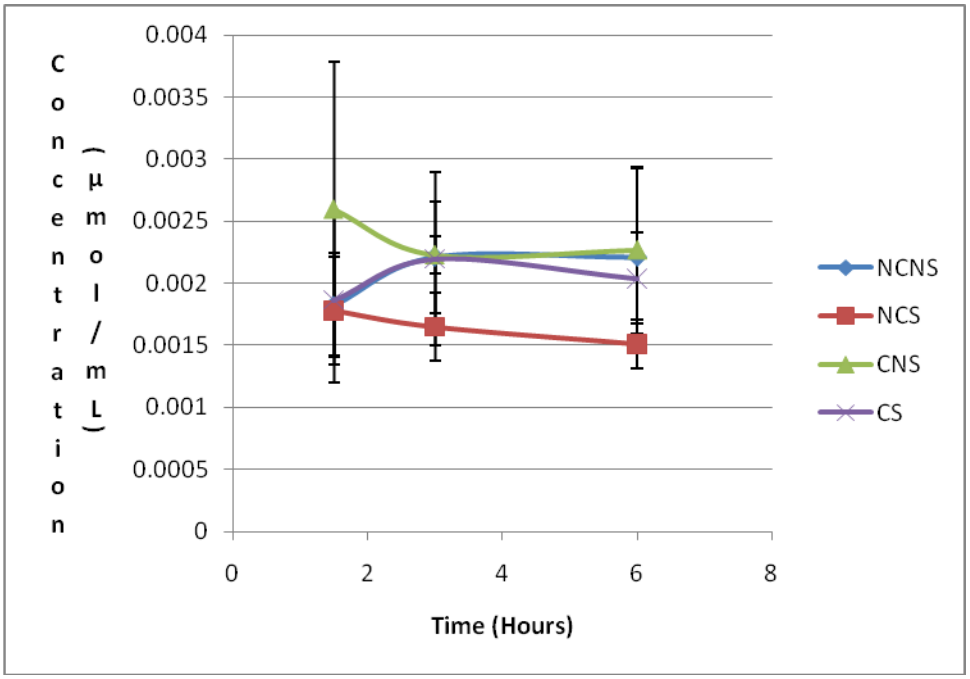
50 µL of the samples were manually injected in to the HPLC maintaining the same chromatographic conditions as used for standard analysis. The equation of the line obtained from the calibration curve was used to calculate the amount of the derivative in µmol. The derivative eluted at around 3.4 minutes (fig 14) when the samples reacted with DTNB were run on HPLC system. Table 12 gives the details of the sample average area peak data (µV*Sec) obtained by averaging the peak area values of seven individual growth curves. The concentration of the derivative in µmol /mL was calculated by multiplying with 1000 and dividing by 0.05 mL (for 50 µL of sample). The result was then divided by 50 (sample was 50 times concentrated) to obtain the concentration in µmol/mL.

.

Table 12. Effect of selenite toxicity on glutathione derivative

Sample	Average peak area (µV*Sec)	Amount of derivative (µmol)	Concentration of derivative (µmol/mL)
NCNS T0	73642.86	0.004557	0.001823
NCS T0	67842.71	0.004448	0.001779
CNS T0	190725.9	0.006496	0.002599
CS T0	80830	0.004665	0.001866
NCNS T1	128580.5	0.005526	0.002210
NCS T1	48075.67	0.004119	0.001648
CNS T1	135270.7	0.005572	0.002229
CS T1	130560	0.005494	0.002197
NCNS T2	132349.3	0.005523	0.002209
NCS T2	27216.57	0.003771	0.001508
CNS T2	146252	0.005672	0.002269
CS T2	106941.3	0.005010	0.00204

Figure 16 Effect of selenite on glutathione concentration. The figure depicts the effect of selenite on the glutathione concentration, where in the concentration (μmol/mL) of the derivative is represented on the y-axis and the time in hours on the x-axis. As seen from the graph, although NCNS, CNS and CS started with different values at TO, the value overlap at T1 and are more or less stable at T2. NCS showed declined from T0 to T2. Error bars were calculated using student t test with 95% confidence interval.


Concentration (µmol/mL)
0.004
0.0035
0.003
0.0025
0.002
0.0015
0.001
0.0005
0
0
2
4
6
8
Time (Hours)
NCNS
NCS
CNS
CS

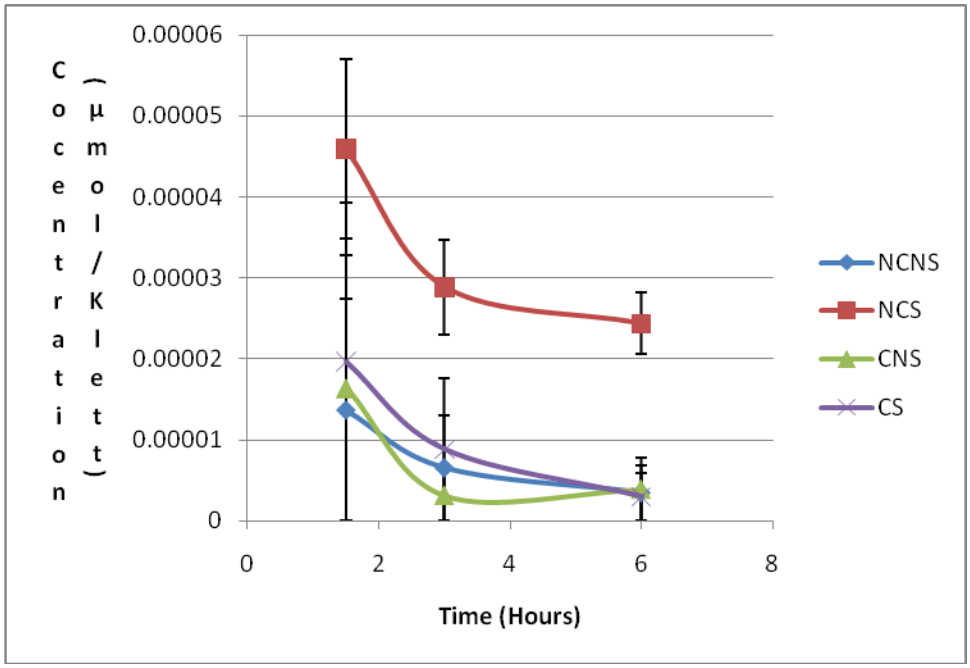
5.7. Normalized glutathione concentration per Klett units

The concentration (µmol/mL) of the derivative produced when the samples were reacted with DTNB was divided by the appropriate Klett measurements to get the value per cell. Table 14 shows the average values of seven individual growth curves.

Table 13. Normalized glutathione concentration data per Klett

Sample	Concentration (µmol/Klett)
NCNS T0	4.578E-05
NCS T0	4.591E-05
CNS T0	5.768E-05
CS T0	4.585E-05
NCNS T1	2.420E-05
NCS T1	2.887E-05
CNS T1	2.306E-05
CS T1	2.298E-05
NCNS T2	1.110E-05
NCS T2	2.436E-05
CNS T2	1.201E-05
CS T2	8.969E-06

Figure 17. Normalized glutathione concentration per Klett. It shows the effect of selenite presence on the glutathione concentration (µmol/Klett unit). Time in hours was plotted on the x-axis and concentration (µmol/Klett unit) on the y- axis. The error bars were calculated using student t test with 95% confidence level.


Concentration (µmol/Kl et t)
0.00006
0.00005
0.00004
0.00003
0.00002
0.00001
0
0
2
4
6
8
Time (Hours)
NCNS
NCS
CNS
CS

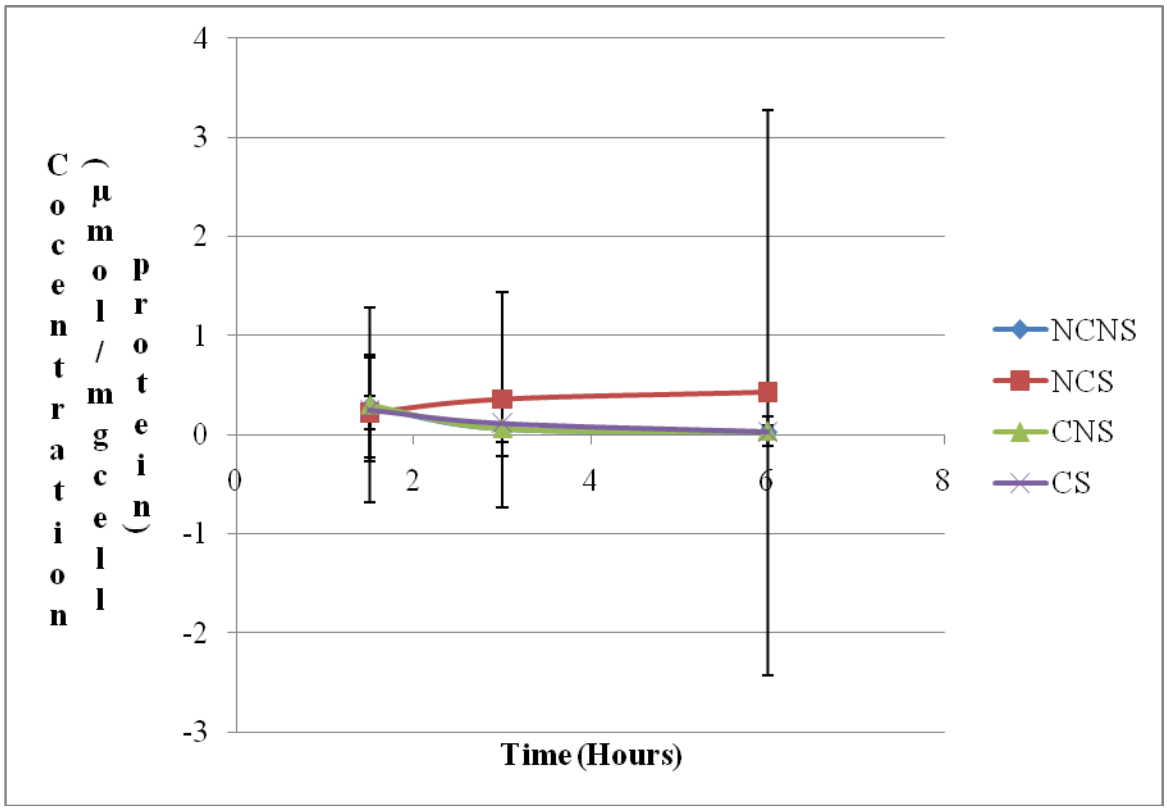
5.8. Normalized glutathione concentration per total cell protein

To further inspect the concentration of the derivative per milligram of cell protein, the HPLC results obtained were divided with Bradford assay data. The table 14 shows the average of two individual growth curves.

Table 14. Normalized glutathione concentration per cell protein

Sample	GSH concentration (μmol/mg cell protein)
NCNS T0	0.2874
NCS T0	0.2189
CNS T0	0.2988
CS T0	0.249513
NCNS T1	0.05735
NCS T1	0.3547
CNS T1	0.06086
CS T1	0.1140
NCNS T2	0.02847
NCS T2	0.4255
CNS T2	0.03217
CS T2	0.02827

Figure 17. Normalized glutathione concentration per cell protein. It represents the derivative concentration (μmol/mg cell protein) on the y-axis and the time in hours on the x-axis. The errors were calculated using student t test with 95% confidence level.


Concentration (µmol/mg cell protein)
4
3
2
1
0
-1
-2
-3
0
2
4
6
8
Time (Hours)
NCNS
NCS
CNS
CS

CHAPTER VI: DISCUSSION

Enterobacter sp. YSU growth curves were performed in the presence and absence of either selenite and cysteine or both. Resulting in four cultures represented as NCNS (No Cysteine, No Selenite), NCS (No Cysteine, Selenite), CNS (Cysteine, No Selenite) and CS (Cysteine, Selenite). Turbidity was measured by Klett colorimeter after every 45 minutes. Selenite (40 mM) or equal volumes of water control were added after 90 minutes of growth in to appropriate flasks. The concentration of selenite was chosen according to the resistance levels of *Enterobacter sp* YSU towards the selenite concentration determined by performing MIC's (Minimum Inhibitory Concentrations). The average Klett measurements of nine growth curves after six hours of growth of NCNS, CNS and CS were 206, 189 and 306 Klett units respectively where as for NCS it was 63 Klett units (Table 5). The results (fig 7) confirmed that *Enterobacter sp* YSU is sensitive to selenite when grown in M-9 minimal medium in the absence of cysteine (NCS) while it is resistant to selenite when grown in M-9 minimal medium in the presence of cysteine (CS).

The experiments were performed to better understand the toxicity mechanism of selenite and cysteine mediated selenite resistance. The assumption that NCS cells experience oxidative stress was based on previous proteomics work that showed that the NCS cells under stress conditions express more stress proteins than CS strains. In addition, RT-PCR was used to show that the NCS cells express the sulfate permease genes at a higher level than the CS cells.[99] This suggests that in the absence of cysteine, selenite enters the cells using the sulfate permease genes, reacts with glutathione and causes oxidative stress.[73] When cysteine is present, cysteine is imported into the cells and indirectly represses the expression of sulfate permease genes by feedback inhibition of

*Cys*E. In this case selenite can only enter the cell through the direct pathway. It is able to enter the cells probably through the specific pathway because the cells turn deep red during stationary phase of growth. During log phase the cells could be pumping out the selenite or excluding it. The hypothesis was tested by measuring the reduced thiol and reduced glutathione levels in cells that were exposed to selenite in the presence and absence of cysteine. Also Bradford assay was performed for determination of total protein concentration which was used to normalize thiol and reduced glutathione levels.

The thiol content in NCS declined markedly compared to thiol content in NCNS, CNS and CS. In the presence of L-cysteine, only a small fraction of RSH content became oxidized. Thus cysteine appears to have induced resistance to oxidative stress by preventing the entrance of selenite through the non specific pathway. Similar results were obtained for Bradford assay and HPLC analysis which resulted in decrease of total protein concentration and the glutathione derivative concentration in NCS respectively. NCNS, CNS and CS had similar results all through suggesting the role of cysteine in selenite resistance. The presence of L-cysteine resulted in resistance towards the selenite. To determine the total reduced thiol content per cell, the RSH (µmol/mg cell protein) and RSH (µmol/ Klett units) was calculated. The data obtained had inconclusive results.

The levels of oxidative stress were determined by measuring the levels of reduced thiols. The thiol concentration in NCNS, CNS and CS seemed to increase as per time while it decreased in NCS. 7.29 µmol/mL, 11.30 µmol/mL and 4.36 µmol/mL were the concentrations of NCNS, CNS and CS respectively after 1.5 hours of addition of selenite or water control (T1). Whereas the concentration NCS was 1.33 µmol/mL at T1. With an increase of 3.07 µmol/mL, 7.11 µmol/mL and 0.725 µmol/mL in NCS, CNS and CS

respectively while a decrease of 4.95 µmol/mL in NCS at T1from T0. NCNS and CNS being the control showed highest increase in thiol concentration while CS showed less increase compared to the control but more compared to NCS which decreased of all. Thus, it can be concluded that in the presence of L-cysteine, only a small fraction of RSH content became oxidized. (fig 10)

Assuming thiol oxidation to have taken place immediately after selenite addition, extra samples were collected, 45 minutes after addition of selenite or water control (labeled as S1) for thiol and Bradford assay (not shown in the results). Samples S1 were collected only for two of the growth curves performed. As there was not much difference seen in the values of S1 and T1 (samples harvested after 1.5 hours of addition of selenite or water control) the data was not included in the results. Samples were also collected at T24 (after 24hours of growth) for Bradford and thiol assay but were not included in the results to be in consistent with HPLC data where T24 was not collected. Furthermore the Klett readings of CS were too high attributing to the fact that it was highly red color due to the formation of elemental selenium and thus the Klett colorimeter showed high values.

In accordance with the thiol assay it was observed that the protein content in NCNS, CNS and CS increased where as the total protein concentration of NCS declined (fig 9). 0.0970 mg/mL, 0.00560 mg/mL, 0.0970 mg/mL and 0.0821 mg/mL were the total protein concentration in NCNS, NCS, CNS and CS respectively at T2 based on the average of three growth curves (table 7). The total protein concentration of NCNS, CNS and CS increased similarly to the same extent while there was a clear decrease in the protein concentration of NCS.

The thiol concentration and Bradford assay results suggest that there is a decrease in RSH content of *Enterobacter* sp. YSU when exposed to selenite in the absence of cysteine. This decrease or thiol oxidation is not just because of cell death but was selenite dependent. As the protein and thiol content of CS which was exposed to same amount of selenite as that of NCS but in presence of cysteine still seemed to increase. Thus, these results confirm cysteine's role in protection against selenite dependent thiol oxidation.

In order to further prove the hypothesis HPLC analysis was performed to specifically quantify glutathione using DTNB. As reduced glutathione is the predominant thiol, its concentration was measured to deduce the effect of selenite toxicity. It has an important role in biological processes such as protecting the cells from reactive oxygen species and free radicals. As DTNB effectively quantifies the concentration of thiols, it was used to get the derivative (mixed disulfide) whose concentration was measured to check the levels of oxidative stress.

Reaction of DTNB with thiol forms mixed disulfide (derivative) and the monomer TNB regardless of the thiol.

$$DTNB + GSH \rightarrow GS\text{-}TNB + TNB$$

The mixed disulfide might have different retention times. The method is however selective as each individual peak of mixed disulfides can be singled out based on the retention times on the chromatogram. In the present method all the analytes were separated.

The elution time of DTNB was confirmed by running a sample consisting of only DTNB which resulted in a single peak at 14.0 minutes. To check the retention time of

reduced glutathione only glutathione (10 mM) was run on HPLC system without DTNB, it showed no peaks except for the one at 18.00 minutes which was seen in all the samples analyzed including blank with its identity being unconfirmed. Thus, in order to confirm the retention time of the derivative increasing concentration of glutathione (0.5 mM – 2.5 mM) with constant amount of DTNB (first 50 µL of 0.5 mM DTNB was added as no color change was observed 10 µL of 5mM DTNB was added). The chromatogram resulted in 4 peaks majorly (the peak at 18.0 minutes was not taken in to consideration as it was expected in all cases). The first peak appeared at 3.0 minutes, the second at 3.3 minutes while the third and fourth at 10.00 minutes and 14.00 minutes respectively. The peak at 14.00 minutes and 3.0 minutes was confirmed to be DTNB and TNB as per previous analysis. While the peak at 3.3 minutes was considered to be due to derivative. The peak at 3.3 minutes was confirmed to be derivative as the intensity/ height of the peak increased with an increase in concentration of the glutathione. While in case of experiments where to the increasing concentrations of glutathione from 0.5 mM – 2.5 mM at least two fold excess of DTNB was added on a molar basis the peaks at 3.0 and 3.3 minutes increased as well with the increase in concentration.

The HPLC results resembled the thiol and Bradford assay. As such, the derivative levels were less in the NCS, attributed to the presence of high oxidative stress, whereas the levels of CNS, CS and NCNS were maintained to a similar extent.

The results showed that derivative levels in CNS started at a higher level at T0 and decreased at T1 and then was maintained to the same level at T2 as T1. The reasons behind its increased levels are unknown. The instrument experienced some problems which included high pressure shutdown, may be column contamination. The samples

used were not filtered before injecting in to HPLC system. Filtration would have given more sharper and sensitive peaks.

In order to normalize the total reduced thiol concentration to total cell protein the total reduced thiol concentration is obtained in µmol/mg cell protein by dividing the thiol assay data with the Bradford assay data. The reduced thiol per total cell protein results showed that in all four cultures it started with a higher value (at T0) and decreased at T1 while it stabilized at T2. NCS had the lowest values as in all cases and the thiol oxidation is attributed to exposure of selenite where as NCNS, CNS and CS had values more than compared to NCS. Still the values were greater for CS compared to NCS. To further clarify the assumption reduced thiol concentration were normalized by dividing with Klett measurements at T0, T1 and T2. The reduced thiol content (µmol) versus Klett units also gave similar results but showed more stability in thiol content at T0, T1 and T2. In all cases NCS showed the least where as CS showed either similar results to that of NCNS and CNS or atleast more amount and resistance compared to NCS. Thus appears that cysteine might have provided resistance from selenite and thus did not result in cell death.

Thus, the thiol assay and Bradford assay results showed that there was a dramatic decrease in reduced thiols and protein content in NCS samples compared to CS samples in consistence with the hypothesis. But when cell number was taken in to account (reduced thiol content versus total protein concentration or Klett units) there was actually not much difference seen in the RSH values of NCS and CS. NCNS, NCS, CNS and CS all started with a higher values at T0 and decreased at T1. The NCS thiol content per cell decreased further at T2 as expected compared to others which were similarly stabilized.

The CS values seemed much closer to NCS values than to the control which was unexpected. The reduced thiol content per milligram cell protein value of NCNS, CNS and CS were 168.2 µmol/mg cell protein, 241.3 µmol/mg cell protein and 124.1 µmol/mg cell protein respectively where as that of NCS was 94.4 µmol/mg cell protein. At T2 the RSH content per cell protein was 179.0 µmol/mg cell protein, 214.0 µmol/mg cell protein and 124.1 µmol/mg cell protein respectively for NCNS, CNS and CS where as 99.5 µmol/g cell protein for NCS. Thus, as per the numbers reduced thiol content per cell protein was maintained in CS, increased in NCS and NCNS while decreased in CNS.

When reduced thiol content was divided by Klett units the concentration of NCNS, CNS and CS at T1 were 0.0774 µmol/cell, 0.106 µmol/cell and 0.0442 µmol/cell respectively and 0.0238 µmol/cell for NCS. At T2 it was 0.0982 µmol/cell for NCNS, 0.102 µmol/cell for CNS, 0.035 µmol/cell for CS and 0.021 µmol/cell for NCS, based on the average values of seven individual growth curves.

As referring to HPLC graph (fig 17) representing the derivative concentration versus Klett unit or the derivative concentration versus milligram cell protein (fig 18). The results for NCNS, CNS and CS were close to each other but the graph showed reverse results from what was expected. There was actually more reduced thiol level in the NCS than the other strains. As seen for the Bradford assay results (fig 9) there was dramatic decrease in the concentration of protein (mg/mL) in NCS strain. This decreased amount was used to divide the decreased amount of thiol content and this might be the reason for increased calculated values. Thus, arising a doubt on the accuracy of the measurement. The increase in NCS values also suggests that the NCS cells are lysing and losing their protein. The cells must have been breaking open, releasing all of their

protein. It would have been more accurate if thiol reading could be obtained by measuring the protein in the cells and in the growth medium and then adding the two protein measurements together. The sum of these could be used to divide the thiol content and obtaining the thiol content per cell value. Similar experiments performed on tellurite toxicity, samples for protein analysis were collected just two times at beginning and at the end of the experimentation period. The use of these values in calculation is ambiguous and the data was not shown. It might have been that the two values were averaged and used for the appropriate strains.[106] There is requirement for more trials especially with regards to Bradford assay whose values were based on only two growth curves. This might be the reason for standard deviation. Also it might be useful to examine the NCS strain of bacteria under a microscope (under the atomic force microscope) to check if the cells are still intact.

The role of cysteine in selenite resistance cannot be disregarded as it showed similar results compared to that of control. Furthermore the thiol content and protein content also remained close to the control in the presence of cysteine. But maybe there is a requirement of more trials and proper procedure for normalization.

Conclusion

All the experiments were performed to better understand the toxicity mechanism of selenite and cysteine mediated selenite resistance. Sensitive cells are killed by reactive oxygen species in the presence of selenite and absence of cysteine in the culture media. The growth curve, thiol, protein and HPLC analysis suggested cysteine provided

resistance relieved oxidative stress. But the normalized levels calculated need's improvised measurements.

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